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Darstellung von *n*-Hexin-1-olen und *n*-Hexen-1-olen

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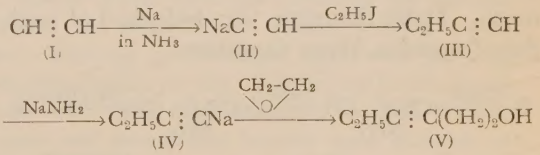
Institut für Chemische Forschung, Universität Kyoto

Eingegangen am 19 Mai 1959

In this paper are described the syntheses of all positional and geometrical isomers of *n*-hexyn-1-ols and *n*-hexen-1-ols. Each of 2-, 3-, 4- and 5-hexyn-1-ol was synthesized from sodium-acetylide in liquid ammonia in higher yield. Then 2-, 3-, 4-*trans*- and 5-hexen-1-ol were obtained by the reduction of corresponding *n*-hexyn-1-ol with sodium in liquid ammonia, and 2-, 3- and 4-*cis*-hexen-1-ol were prepared by the partial hydrogenation at low temperature using palladium-bariumsulfate catalyst. These alcohols were purified through the 3,5-dinitrobenzoates. The infra-red spectra of above compounds were discussed.

Der Blätteralkohol, ein Isomerer von *n*-Hexen-1-olen, ist die Hauptkomponente esd eigentümlichen Geruches in den Blättern und kommt weit in den frischen Blättern der meheren Pflanzen in freiem sowie gebundenem Zustand verbreiten vor. Dieser Alkohol, der das Bruchstück von Jasmon[1], Veilchenblätteraldehyd[2] und Gurkenalkohol[3] ist, ist von alters her von pflanzphysiologischem sowie riechstoffchem Gesichtspunkte aus und auch von geometrischem Interesse aus untersucht worden. Vor kurzem sind die Untersuchungen über die Lockwirkungseigenschaften der *n*-Hexen-1-ole hier und da ausgenommen[4~6]. Es handelt sich daher um die reizvolle Eigenschaften der *n*-Hexen-1-ole. Aber da es in theoretisch möglichen sieben geometrischen Stellungsisomeren die unbekanten Isomere und auch in den bereits dargestellten Stoffen die geometrisch unreinen sind, haben wir *all* sieben Isomere von *n*-Hexen-1-olen besonders in reiner Form hergestellt. Dafür haben wir erst vier Isomere von *n*-Hexin-1-olen dargestellt und dann diese *n*-Hexen-1-ole durch die Hydrierung der entsprechenden *n*-Hexin-1-ole gewonnen.

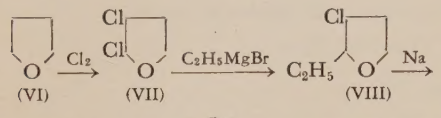
Zur Gewinnung des 3-Hexin-1-ols (V) musste man früher das sehr müsame Verfahren benutzen[7~9]. Nachdem konnte F. Sondheimer[10] aber von Acetylen aus 3-Hexin-1-ol in guter Ausbeute darstellen. Wir haben 3-Hexin-1-ol nach den Verbesserungen dieser Methode gewonnen. Aber in diesem Fall fanden wir, dass die ziemliche Menge von den sekundären



Alkoholen bei der Reaktion des 1-Butin-natriums mit Äthylenoxyd und auch von dem Alkoxyäthanol bei dem Gebrauch von überschüssiger Menge des Äthylenoxyds entstehen kann. Daher unter der vorsichtigen Wahl der Tropfenmengen von Äthylenoxyd und der Reaktionstemperatur wurde dieser Versuch wiederholt. Aus diesen Versuchen stellten wir das 2-, 4- und 5-Hexin-

1) W. Treff und H. Werner, *Ber.*, **66**, 1521 (1933); **68**, 640 (1935). L. Ruzicka und M. Pfeiffer, *Helv. Chim. Acta*, **16**, 1208 (1933). S. H. Harper und R.J.D. Smith, *J. Chem. Soc.*, **1955**, 1512.
2) S. Takei und M. Ohno, *J. Agr. Chem. Soc. Japan*, **15**, 193 (1939).
3) S. Takei, M. Ohno und Mitarbeiter, *J. Agr. Chem. Soc. Japan*, **14**, 717 (1938).
4) T. Watanabe, *Nature*, **182**, 325 (1958).
5) A. Butenandt, *Naturwissenschaftliche Rundschau*, **12**, 463 (1955).
6) A. Butenandt, E. Hecker und H. G. Zachau, *Ber.*, **88**, 1185 (1955).
7) M. Stoll und A. Rouve, *Helv. Chim. Acta*, **21**, 1542 (1938).
8) S. Takei, M. Ohno und K. Sinosaki, *Ber.*, **73**, 950 (1940).
9) L. Crombie und S. H. Harper, *J. Chem. Soc.*, **1950**, 1714.
10) F. Sondheimer, *J. Chem. Soc.*, **1950**, 887.

1-ol dar. Ausserdem konnten wir auch durch die Zusammenfügung der Verfahren von L. Crombie[9] und S. Takei[8] 3-Hexin-1-ol gewinnen.



(VI) (VII) (VIII)

$$C_2H_5CH{=}CH(CH_2)_2OH \xrightarrow{Br_2} \underset{(IX)}{C_2H_5(CHBr)_2(CH_2)_2OH} \xrightarrow{KOH} \underset{(V)}{C_2H_5C \vdots C(CH_2)_2OH}$$

Wir stellten 2-Hexin-1-ol (XIII) nach dem Verfahren von M. S. Newman[11] dar. Das durch diesen Weg erhaltene 2-Hexin-1-ol gab das schlechte Ergebnisse in der Reinheit.

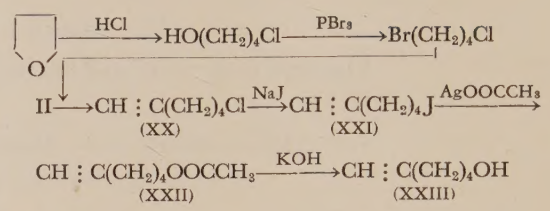
$$II \xrightarrow{C_3H_7Br} \underset{(XI)}{C_3H_7C \vdots CH} \xrightarrow{C_2H_5MgBr} \underset{(XII)}{C_3H_7C \vdots CMgBr} \xrightarrow{HCHO} \underset{(XIII)}{C_3H_7C \vdots CCH_2OH}$$

4-Hexin-1-ol (XIX) wurde erstmal von L. Crombie[12] nur in geringer Ausbeute erhalten. Obgleich M. S. Newman[11] zur Gewinnung des 4-Hexin-1-ol-acetats die Umsetzung von 1-Jod-4-hexin mit Kaliumacetat versuchte, gelang es ihm nicht. Wir konnten jedoch statt Kaliumacetats durch die Benutzung von Silberacetat in guter Ausbeute dieses 4-Hexin-1-ol-acetat gewinnen. Daher konnten wir 4-Hexin-1-ol nach dem folgenden Wege darstellen.

$$II \xrightarrow{CH_3Br} \underset{(XIV)}{CH_3C \vdots CH} \xrightarrow{NaNH_2} \underset{(XV)}{CH_3C \vdots CNa} \xrightarrow{Br(CH_2)_3Cl} \underset{(XVI)}{CH_3C \vdots C(CH_2)_5Cl} \xrightarrow{NaJ} \underset{(XVII)}{CH_3C \vdots C(CH_2)_3J} \xrightarrow{AgOOCCH_3} \underset{(XVIII)}{CH_3C \vdots C(CH_2)_3OOCCH_3} \xrightarrow{KOH} \underset{(XIX)}{CH_3C \vdots C(CH_2)_3OH}$$

5-Hexin-1-ol (XXIII) konnten wir durch den analoge Weg bei 4-Hexin-1-ol darstellen.

Solch stellten wir alle vier Isomere über das in flüssigem Ammoniak gelösten Acetylennatrium (II) dar. Besonders ist diese Darstellung



von 4- und 5-Hexin-1-ol in der Ausbeute bedeutend überlegen als bisher bekannte Methoden. Reines *n*-Hexin-1-ol als Ausgangsmaterial für die Darstellung des *trans*- oder *cis*-Hexen-1-ols wurde über 3,5-Dinitrobenzoat oder Phthalsäurehalbester in reiner Form gewonnen. In den sieben geometrischen Isomeren des *n*-Hexen-1-ols wurden *trans*-Isomere durch die Reduktion des entsprechenden *n*-Hexin-1-ols mit dem in flüssigem Ammoniak gelösten Natrium und dagegen *cis*-Isomere durch die katalytische partielle Hydrierung in gegenwart von Palladium-Bariumsulfat bei $-15°$ gewonnen.

Betrachtungen über Infrarotspektren

Von besonderem Interesse für die Stellungszuordung ist ein Vergleich der Infrarotspektren der vier *n*-Hexin-1-ole. In Tafel I ist das charakteristische Band der vier *n*-Hexin-1-ole gezeigt worden. In diesen Banden wurde nur

TAFEL I

		RC⋮CR′	RC⋮CH
$C_3H_7C \vdots CCH_2OH$	(XIII)	4.46 μ	—
$C_2H_5C \vdots C(CH_2)_2OH$	(V)	4.51	—
$CH_3C \vdots C(CH_2)_3OH$	(XIX)	4.45	—
$CH \vdots C(CH_2)_4OH$	(XXIII)	—	4.65 μ

die *RC*⋮*CR*-Valenzschwingung von dem 3-Hexin-1-ol zwischen 4.4～4.8 μ kaum gefunden. Es scheint darauf zu begründen, dass die Molekularstruktur des 3-Hexin-1-ols für die dreifache Bindung fast symmetrisch ist. Die Tatsache entspricht der von Wotiz[13] erhaltenen Betrachtung über die symmetrischen Alkynverbindungen. 2-Hexin-1-ol zeigt bei 4.46 μ ein scharfes Band und 4-Hexin-1-ol bei 4.45μ ein schwaches Band. Aber für die *HC*⋮*CR*-Valenzschwindung von dem 5-Hexin-1-ol wird ein charakteristisch scharfes Band bei 4.65 μ gefunden.

11) M. S. Newman und J. H. Wotiz, *J. Am. Chem. Soc.*, **71**, 1292 (1949).
12) L. Crombie und S. H. Harper, *J. Chem. Soc.*, **1950**, 1707.
13) J.H. Wotiz und F. A. Miller, *J. Am. Chem. Soc.*, **71**, 3441 (1949).

Wir finden bei 10.3μ das charakteristische Band für *trans*-Hexen-1-ole und bei 11.5μ und 13.8μ das für *cis*-Hexen-1-ole. Aber in *cis*-Isomeren wird nur 3-*cis*-Hexen-1-ol das sehr schwache Band von der $-\underset{H}{C}=\underset{H}{C}-$ Valenzschwindung bei 10.3μ gefunden. Wir können den genauige Grund für diese Tatsache nicht erfinden und aber weiter wollen über das Problem untersuchen.

BESCHREIBUNG DER VERSUCHE

I) Darstellung von den vier Stellungsisomeren des *n*-Hexin-1-ols.

3-Hexin-1-ol (V)

900 ccm Flüssiges Ammoniak wurden in dem voraus genug getrockneten und bei $-40°$ gekühlten Kolben eingegossen und unter Rührung das genug gereinigte, getrocknete und gekühlte Acetylen wurde eingeleitet. Mit der Einleitung wurden 25 g Natrium in kleinen Stück am jeden Entfärben des Blaues mit der Reaktionsflüssigkeit versetzt. Sobald nach dem Zugaben eines letzten Natriumstücks die blaue Farbe des Gemisches verschwindet, wurde die Einleitung unterbrochen und hierauf 15 Minuten wurde das Gemisch gerührt. 155 g Äthyljodid in 150 ccm Äther wurden während 1 Stundes langsam getropft und bei $-40°$, 5~6 Stunden gerührt. Ohne das so erhaltene 1-Butin aus flüssigem Ammoniak zu isolieren, wurde das Natriumamid aus 25 g Natrium, 900 ccm flüssigem Ammoniak und 1g Ferri-nitrat teilweise mit Reaktionsgemisch versetzt. Nach 1 Stunde wurden 95 g Äthylenoxyd sofort eingegossen und das Gemisch wurde bei $-40°$, 24 Stunden gerührt. 60g Ammoniumchlorid wurden versetzt und flüssiges Ammoniak wurde zunächst bei Raumtemperatur und dann auf dem Wasserbad verdampft. Nach das Reaktionsprodukt in Warser gelöst wurde und ausgeäthert, ging 3-Hexin-1-ol bei 163~7° über. n_D^{20} 1.4542, Ausb. 47 g (48% des angewandten Äthyljodids). 3,5-Dinitrobenzoat: Schmp. 71°, $C_{13}H_{12}O_6N_2$ (292.25) Ber. C 53.46, H 4.14, N 9.56. Gef. C 53.26, H 4.17, N 9.45.

Reines 3-Hexin-1-ol: 3-Hexin-1-ol-3, 5-dinitrobenzoat wurde mit alkohol. Kali (5 g Kalium-hydrat, 5 ccm Wasser und 20 ccm Alkohol) versetzt und reines 3-Hexin-1-ol wurde durch Wasserdampf gewonnen. Sdp. 163°, n_D^{20} 1.4537. Auch aus Phthalsäure-halbester. öligen Zustand, wurde reines 3-Hexin-1-ol erhalten. Sdp. 162 ~4°, n_D^{20} 1.4573.

2-Äthyl-3-chlortetrahydrofuran (VIII): 262g 2, 3-Dichlortetrahydrefuran aus Tetrahydrofuran (VI) mit Chlorgas wurden im Grignard'sche Reagens aus 240 g Äthylbromid und 52.6 g Magnesiumspäne langsam eingegossen. Nach der gewöhnlichen Behandlung ging 2-Äthyl-3-chlor-tetrahydrofuran bei 62~75°/21 mm über. Ausb. 174 g (69% d. Th.).

3-*cis*, *trans*-Hexen-1-ol (IX): 174 g 2-Äthyl-3-chlortetrahydrofuran wurden unter Rührung in Natriumpulver in Äther langsam eingegossen. Nachdem wurde das Gemisch unter Rückfluss gekocht und mit dem Verschwinden des Natriumpulvers wurde das Reaktionsprodukt mit 300 g Eisstück versetzt. 3-*cis*, *trans*-Hexen-1-ol ging bei 64~66°/19 mm über. Ausb. 109.5 g (71% d. Th.).

3,4-Dibrom-hexan-1-ol (X): 20 g 3-*cis*, *trans*-Hexen-1-ol wurden in 200 ccm Äther unter Eiskühlung mit 32 g Brom behandelt. Nach dem Abdampfen des Äthers wurde das Reaktionsprodukt fraktioniert. Der Hauptteil siedet bei 115~122°/5 mm. Ausb. 41 g (78.8% d. Th.).

Rohes 3-Hexin-1-ol (V): 160g 3,4-Dibrom-hexan-1-ol wurden mit alkohol. Kali (80g Kalium-hydrat, 5 ccm Wasser und 160 ccm Alkohol) versetzt, wobei sich Kalium-bromid ausschied. Es wurde auf dem Wasserbad unter Rückfluss einigen Stunden gekocht. Das ausgeschiedene Kalium-bromid wurde mit Wasser abgelöst und das Reaktionsprodukt wurde ausgeäthert. 3-Hexin-1-ol siedete bei 163~172°. Dieses 3-Hexin-1-ol zeigt in der Flamme keine Halogen-Reaktion. 3, 5-Dinitrobenzoat: Schmp. 73°. Reines 3-Hexin-1-ol: Sdp. 72°/20 mm, n_D^{20} 1.4535.

2-Hexin-1-ol (XIII)

42 g 1-Pentin (85% des angewandten Propylbromides) in 210 ccm Äther, durch das Umsetzung von Acetylennatrium mit Propylbromid erhalten wurden, wurden unter Rührung und Rückfluss tropfenweise mit Grignard'sche Reagens aus 84 g. Äthylbromid in 100 ccm abs. Äther und 18.5 g Magnesiumspäne in 210 ccm Äther versetzt und hierauf 5~6 Stunden unter Rückfluss erwärmt. Nachdem wurde der Tropftrichter vom Einleitungsrohr ersetzt. Der inner Durchmesser des Einleitungsrohrs ist 8 mm und dieses Ende muss nahe an der Oberfläche der Flüssigkeit stellen lassen. Unter Rührung und Rückfluss wurde das bei 180~200° entstehende Formaldehyd (Paraformaldehyd muss 2 Wochen auf dem Phosphorsäureanhydrid getrocknet werden) mit dem Strom des Stickstoffes eingeleitet. Nach der gewöhnlichen Behandlung, ging 2-Hexin-1-ol bei 160~173° über. n_D^{20} 1.4535, Ausb. 40 g (67% d. Th.). 3,5-Dinitrobenzoat: Schmp. 68.5°, $C_{13}H_{12}O_6N_2$ (292.25) Ber. C 53.46, H 4.14, N 9.59 Gef. C 53.62, H 4.22, N

9.44. Pathalsäurehalbester: Schmp. 83°. Reines 2-Hexin-1-ol: Sdp. 167°, n_D^{20} 1.4551.

4-Hexin-1-ol (XIX)

1-Chlor-4-hexin (XVI): 257 g Methylbromid wurden mit dem in 1500 ccm flüssigem Ammoniak gelösten Acetylen-natrium aus 36 g Natrium versetzt und hierauf 5~6 Stunden bei −40° gerührt. Das Natriumamid aus 36 g Natrium 1000 ccm flüssigem Ammoniak und 1g Ferri-nitrat wurde mit dem in flüssigem Ammoniak gebildeten 1-Propin versetzt und hierauf 1 Stunde bei −40° gerührt. 228 g 1-Brom-3-chlor-propan wurden im 1-Propin-natrium langsam eingetropft und dann wurde das Gemisch bei −40°, 10 Stunden gerührt. 95 g Ammoniumchlorid wurden vorsichtig versetzt. Nach dem Abdampfen des flüssigen Ammoniaks ging 1-Chlor-4-hexin bei 146~158° über. Ausb. 70 g (40% d.Th.).

1-Jod-4-hexin (XVII): 65 g 1-Chlor-4-hexin wurden mit 95 g Natriumjodid in 300 ccm Aceton versetzt und auf dem Wasserbad unter Rührung und Rückfluss 20 Stunden gekocht. 1-Jod-4-hexin wurde bei 97~103°/35 mm erhalten. Ausb. 109 g (94% d.Th.).

4-Hexin-1-ol-acetat (XVII): 34.3 g 1-Jod-4-hexin wurde mit 42.5 g Silberacetat in 200 ccm Benzol versetzt und auf dem Wasserbad unter Rührung und Rückfluss 5 Stunden gekocht. Nach dem ausgeschiedene Silberjodid filtriert wurde, wurde das Reaktionsprodukt ausgeäthert. 4-Hexin-1-ol-acetat ging bei 96~98°/28 mm über. n_D^{20} 1.4526, Ausb. 18.7 g (78.7% d.Th.)

4-Hexin-1-ol (XIX): 17 g 4-Hexin-1-ol-acetat wurden mit 2N alkohol. Kalium-hydrat versetzt und auf dem Wasserbad unter Rückfluss 2 Stunden gekocht. 4-Hexin-1-ol wurde bei 94~97°/30 mm gewonnen. Ausb. 9.2 g (75% d. Th.).

3,5-Dinitrobenzoat: Schmp. 71~71.5°, $C_{13}H_{12}O_6N_2$ (292.25) Ber. C 53.46, H 4.14, N 9.59 Gef. C 53.49, H 4.39, N 9.62. Reines 4-Hexin-1-ol: Sdp. 94~94.7°/30 mm, n_D^{20} 1.4588.

5-Hexin-1-ol (XXIII)

4-Chlorbutan-1-ol: Der Strom des HCl-gases wurden in 72 g Tetrahydrofuran mit 0.1 g Aluminiumchlorid unter Rückfluss bei 65° eingeleitet. Mit dem Anfang der Reaktion steigt die Reaktionstemperatur und nach 4 Stunden bei 105° wurde die Einleitung unterbrochen. 4-Chlorbutan-1-ol ging bei 93~103°/30 mm über. Ausb. 75 g (69% d. Th.).

1-Brom-4-chlorbutan: 95 g Phosphortribromid wurden in 75 g 4-Chlorbutan-1-ol unter Eiskühlung tropfenweise versetzt und 4~5 Stunden gekocht. 1-Brom-4-chlorbutan siedete bei 82~87°/34 mm, 171~176°. Ausb. 94.7 g (80% d.Th.).

1-Chlor-5-hexin (XX): 90 g 1-Brom-4-chlorbutan wurden bei −40° mit Acetylen-natrium aus 12 g Natrium und 500 ccm flüssigem Ammoniak tropfenweise versetzt und hierauf 7 Stunden bei −35° gerührt. Durch gleiches Verfahren bei 1-Chlor-4-hexin wurde 1-Chlor-5-hexin bei 144~148° gewonnen. Ausb. 33.8 g (55.3% d. Th.).

1-Jod-5-hexin (XXI): 29.5 g Chlor-5-hexin wurden mit 50 g Natrium-jodid in 150 ccm Aceton versetzt und durch gleiches Verfahren bei 1-Jod-4-hexin wurde 1-Jod-5-hexin bei 94~98°/38 mm erhalten. n_D^{20} 1.5229, Ausb. 30 g (69.1% d. Th.).

5-Hexin-1-ol-acetat (XXII): 28.5 g 1-Jod-5-hexin wurden mit 22.9 g Silberacetat in 150 ccm Benzol versetzt und auf dem Wasserbad unter Rückfluss 3 Stunden gekocht. 5-Hexin-1-ol-acetat siedet bei 94~96°/32 mm. n_D^{20} 1.4453, Ausb. 14 g (72.9% d. Th.).

5-Hexin-1-ol (XXIII): 13 g 5-Hexin-1-ol-acetat wurden mit 2N alkohol. Kalium-hydrat versetzt und durch gleiches Verfahren wie bei 4-Hexin-1-ol wurde 5-Hexin-1-ol bei 89~91°/30 mm gewonnen. n_D^{20} 1.4457, Ausb. 6.4 g (70.5% d. Th.). 3,5-Dinitrobenzoat: Schmp. 57°, $C_{13}H_{12}N_6O_2$ (292.25) Ber. C 53.42, H 4.14, N 9.59, Gef. C 53.49, H 4.23, N 9.76.

II) Darstellung von sieben geometrischen Stellungsisomeren des *n*-Hexen-1-ols.

***trans*-Isomer**

3-*trans*-Hexen-1-ol: 4.9 g Reines 3-Hexin-1-ol wur-

TAFEL II

Isomer	Sdp. °C	3, 5-Dinitrobenzoat, Schmp. °C		Elemental analyse		
			Ber.	C 53.10	H 4.79	N 9.52
2-*trans*-	153	61.5	Ger.	C 53.32	H 4.74	N 9.26
2-*cis*-	153~6	51~51.5		53.34	4.94	10.00
3-*trans*-	152~4	47		53.32	4.62	9.38
3-*cis*-	157	49.5		53.08	4.83	9.38
4-*trans*-	161~3	33~33.5		53.35	4.83	
4-*cis*-	157~8	25~25.5		52.82	4.83	
5-	157~8	50~1		53.43	4.75	9.45

den in Natrium-flüssigesammoniak-lösung aus 4.6 g Natrium und 200 ccm flüssigem Ammoniak unter Kühlung bei −40° und Rührung eingetropft. Nach der Rührung 2 Stunden entfärbt sich das Blau des Gemisches mit Zugabe von 15 g Ammoniumchlorid. 3-*trans*-Hexen-1-ol siedete bei 150～152°, n_D^{20} 1.4419, Ausb. 3.9 g (80% d. Th.). 3, 5-Dinitrobenzoat: Schmp. 47°. Reines 3-*trans*-Hexen-1-ol: Sdp. 153°, n_D^{20} 1.4361.

Reines 2-, 4-*trans*- und 5-Hexen-1-ol wurden durch das gleiche Verfahren erhalten.

***cis*-Isomer**

3-*cis*-Hexen-1-ol: 2 g Reines 3-Hexin-1-ol in 60 ccm Äther wurden mit 0.8 g Palladium-Bariumsulfat versetzt und mit einer Kaltemischung auf −15° gekühlt. Durch Einleitung vom Wasserstoff im Kolben wurde reines 3-Hexin-1-ol hydriert. In 20 Minuten nahmen 2 g 3-Hexin-1-ol 472 ccm Wasserstoff auf, wonach wurde die Einleitung sofort unterbrochen. 3, 5-Dinitrobenzoa: Schmp. 49.5°. Reines 3-*cis*-Hexen-1-ol: Sdp. 157°.

Reines 2-, 4-*cis*-Hexen-1-ol wurden durch das gleiche Verfahren erhalten. Die physikalischen Konstante und Elementalanalyse von sieben Isomeren des *n*-Hexen-1-ols wurden in Tafel II gezeigt.

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Synthesis of Chromanochromanone and 2-Substituted Isoflavanones

(Synthesis of Rotenoids I)

By Hiroshi Fukami, Syozo Takahashi, Kazuo Konishi and Minoru Nakajima

Dapartment of Agricultural Chemistry, Kyoto University

Received May 26, 1959

Six new 2-substituted isoflavanones were obtained by catalytic hydrogenation of the corresponding isoflavones. Under the same condition, chromenochromon was reduced to chromanochromanone which was the main skeletal unit of rotenoids. The steric configuration of these compounds is discussed.

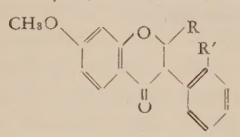
Rotenone and its related compounds are generically called as rotenoids, which contain chromanochromanone (VII) as their main skeletal unit. The chromanochromanone unit has been supposed to have intimate relation with the insecticidal activity of rotenoids. T. R. Seshadri and S. Varadarajan[1] presumed for the biogenesis of the unit to consist in the formation of the chroman ring by dehydration between the two hydroxyl groups of 2-hydroxymethyl-2′-hydroxyisoflavanone. The present paper deals with preparation of 2-substituted isoflavanones and chromanochromanone by means of catalytic hydrogenation.

Six new isoflavanones (I–VI) in the table were obtained by hydrogenation of the corresponding isoflavones[2], and reacted with 2, 4-dinitrophenylhydrazin to give the hydrazones, whilst the isoflavones did not react with the reagent. These isoflavanones show almost identical UV-spectra with each other, having the

1) T. R. Seshadri and S. Varadarajan, *Proc. Indian Acid. Sci.*, **37A**, 784 (1953).

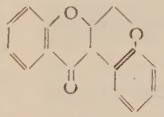
2) Of these isoflavones, 2-hydroxymethyl-7-methoxy- and 2-hydroxymethyl-7,2′-dimethoxy-isoflavone have not been reported and were prepared by bromination followed by hydroxylation of the corresponding 2-methyl-isoflavones.

Isoflavanone		m.p.	m.p. of 2,4-Dinitrophenyl hydrazone	The carbonyl band in IR-spectra	
				Isoflavanone	Original isoflavone
2-Methyl-7-methoxy-	(I)	122°～122.5°	200°	1667 cm^{-1}	1623 cm^{-1}
2-Carbomethoxy-7-methoxy-	(II)	140°～141°	240°～241°	1678	1653
2-Carbethoxy-7-methoxy-	(III)	100°～101°	230.5°～232°	1678	1654
2-Phenyl-7-methoxy-(2,3-diphenyl-7-methoxychromanone)	(IV)	128°～129°	254°～255°	1678	1629
2-Hydroxymethyl-7-methoxy-	(V)	156°～158°	203°～204°	1672	1623
2-Hydroxymethyl-7,2′-dimethoxy-	(VI)	179°～181°	249°～251°	1653	1626



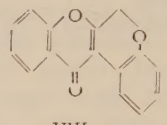
I～VI

I; R=CH_3, R′=H
II; R=$COOCH_3$, R′=H



VII

III; R=$COOC_2H_5$, R′=H
IV; R=C_6H_5, R′=H



VIII

V; R=CH_2OH, R′=H
VI; R=CH_2OH, R′=OCH_3

maxima at 275 mμ (log ε, 4.1) and 310mμ (log ε, 3.8), and exhibit strong carbonyl bands in 1650～1680 cm^{-1} region, which are displaced by about 30～50 cm^{-1} to higher frequency than that of the original isoflavones.

Chromenochromon (VIII) is the main skeletal unit of dehydrorotenone and may be regarded as a derivative of 2′-hydroxyisoflavone. F. B. LaForge[3] prepared chromenochromon by interaction of 2,2′-dihydroxydesoxybenzoin and ethyl bromoacetate in the presence of sodium alcoholate. However, it has not been reported that chromanochromanone is derived from chromenochromon by hydrogenation or any other method. In the present work, chromenochromon was reduced catalytically to give rise to the compound, $C_{16}H_{12}O_3$, m.p. 162°～163°, of which carbonyl band appeared at 1695 cm^{-1} accompanied with the shift by 50 cm^{-1} to higher frequency than that of chromenochromon. By dehydrogenation with iodine the compound gave original chromenochromon. Therefore, the compound was established to be chromano-(3′, 4′, 2, 3)-chromanone (VII).

Chromanochromanone as well as 2-substituted isoflavanones should allow two stereoisomers, i.e. *cis*- and *trans*-isomer, to exist owing to the steric relationship between the substituents at the 2- and the 3-position on the chromanone ring. H. A. Offe[4] obtained another isomer of 2,3-diphenyl-7-methoxychromanone by condensation of resorcinol with α-phenyl cinnamic acid in hydrogen fluoride and the following methylation. By using borofluoride in place of hydrogene fluoride we also prepared the isomer, m. p. 174°～175°, of which melting point was depressed on admixture with the isomer (IV) prepared by catalytic hydrogenation. Taking account of the fact that the catalytic hydrogenation of double bonds in flavones proceeds by *cis*-addition[5], it is reasonable that the isomer (IV) should have *cis*- and consequently another isomer (m.p. 174～175°) have *trans*-configuration. By analogy, chromanochromanone and 2-substituted isoflavanones obtained by catalytic hydrogenation seem to be *cis*-isomers.

EXPERIMENTAL*

2-Hydroxymethyl-7-methoxyisoflavone :

2-Methyl-7-methoxyisoflavone, m.p. 135～136°[6], (5 g), N-bromosuccinimide (3.4 g) and benzoyl peroxide (200 mg) in carbon tetrachloride (250 ml) were refluxed for twenty hours. After cooling, the precipitate was filtered

3) F. B. LaForge, *J. Am. Chem. Soc.*, **54**, 3377 (1932).
4) H. A. Offe, *Chem. Ber.*, **80**, 452 (1947).
5) E. F. King and J. W. Clark-Lewis, *J. Chem. Soc.*, **1955**, 3384; A. Kasahara, *J. Chem. Soc. Japan*, **80**, 416 (1959).
* Melting points were not corrected. IR- and UV-spectra were taken in nujiol and in ethanol respectively.
6) W. Baker and R. Robinson, *J. Chem. Soc.*, **1925**, 1981.

off and the filtrate was evaporated to a yellow syrup, which was recrystallized from ethanol to give 2-bromomethyl-7-methoxyisoflavone, rhombic plates, m.p. 159~160°, (4.3 g). *Anal.* Found : C, 58.74 ; H, 4.02 ; Br, 23.33. Calcd for $C_{17}H_{13}O_3Br$: C, 59.14 ; H, 3.80 ; Br, 23.15.

The mixture of the bromide (1.5 g) and finely powdered silver acetate (3 g) in acetic anhydride (15 ml) was refluxed for six hours on an oil bath, then cooled, filtered to remove insoluble silver salts and the filtrate was poured over crushed ice, left in the refrigerator overnight. The separated brown oil was extracted with ether and the ethereal solution was washed with water and dried over anhydrous sodium sulfate. Removal of ether gave an oil, which was crystallized from aqueous methanol to yield 2-acetoxy-7-methoxyisoflavone, colorless, needles m.p. 111~112° (0.95 g). *Anal.* Found : C, 70.53 ; H, 4.77. Calcd for $C_{19}H_{16}O_5$: C, 70.36 ; H, 4.98.

The acetate (800 mg) was dissolved in ethanol (15 ml) and 2N-sodium-carbonate (5 ml) was added. The solution was refluxed for two hours on a water bath and neutrallized with dil. hydrochloric acid, diluted with water and allowed to stand overnight. The precipitate was collected and recrystallized from ethyl acetate to give 2-hydroxymethyl-7-methoxyisoflavone, pale yellow prisms, m. p. 168° (650 mg). *Anal.* Found : C, 72.45 ; H, 5.19. Calcd for $C_{17}H_{14}O_4$: C, 72.33, H, 5.00. λ_{max}, 235 mμ (log ε, 4.37), 296 mμ (log ε, 4.07).

2-Hydroxymethyl-7, 2′-dimethoxyisoflavone :

The compound was prepared from 2-methyl-7, 2′-dimethoxyisoflavone according to the method described above. 2-Methyl-7, 2′-dimethoxyisoflavone[1] (3.3 g), N-bromosuccinimide (2.4 g) and benzoyl peroxide (150 mg) in carbon tetrachloride (150 ml) yielded crude 2-bromomethyl-7, 2′-dimethoxyisoflavone on reflux for six hours. Recrystallization from *n*-hexane gave colorless prisms, m.p. 105~107° (1.2 g). *Anal.* Found : C, 57.58 ; H, 3.91 ; Br, 19.91. Calcd for $C_{18}H_{15}O_4Br$: C, 57.61; H, 4.03 ; Br, 21.30.

The crude bromide (8.2 g) and silver acetate (15 g) in acetic anhydride (150 ml) were refluxed for seven hours, yielded 2-acetoxymethyl-7, 2′-dimethoxyisoflavone as a brown oil. The crude acetate in ethanol (100 ml) was hydrolized with conc. hydrochloric acid (100 ml) to give an oil, which was chromatographed on alumina with a mixture of ethyl acetate and hexane (1 : 1). The elute yielded 2-hydroxymethyl-7,2′-dimethoxyisoflavone, which was recrystallized from ethyl acetate, pale yellow prisms, m.p. 160~162° (1.7 g). *Anal.* Found : C, 69.58 ; H, 5.42. Calcd for $C_{18}H_{16}O_5$: C, 69.22 ; H, 5.16. λ_{max}, 284 mμ (log ε, 4.11).

Catalytic hydrogenation of isoflavones :

The isoflavanones were obtained according to the following procedure. The mixture of the isoflavone and platinum oxide (about 10% by weight to the isoflavone) in glacial acetic acid was shaken in the atmosphere of hydrogen until 1.2 mole of hydrogen was absorbed. After separation of the catalyst the solvent was removed under reduced pressure and recrystallization of the residue gave the isoflavanone.

2-Methyl-7-methoxyisoflavanone (I) :

2-Methyl-7-methoxyisoflavone, m.p. 135~136°, (2.3 g) and platinum oxide (250 mg) in acetic acid (150 ml) yielded I (0.9 g), which was recrystallized from methanol to give colorless scales, m. p. 122~122.5°. *Anal* Found : C, 75.95 : H, 6.05. Calcd for $C_{17}H_{16}O_3$: C, 76.10 ; H, 6.01. λ_{max}, 235 mμ (log ε, 4.03), 276 mμ (log ε, 4.13), 311 mμ (log ε, 3.88).

The 2, 4-dinitrophenylhydrazone*, m. p. 200°, red needles. *Anal* Found : C, 61.54 ; H, 4.47 ; N, 12.55. Calcd for $C_{23}H_{20}O_6N_4$: C, 61.60 ; H, 4.50 ; N, 12.50.

2-Carbomethoxy-7-methoxyisoflavanone (II) :

2-Carbomethoxy-7-methoxyisoflavone, m.p. 141~142°, (460 mg) and platinum oxide (50 mg) in acetic acid (50 ml) yielded II, which was recrystallized from methanol to give colorless rhombic plates (380 mg). m. p. 140~141°, of which melting point was depressed on admixture with the starting isoflavone. *Anal.* Found : C, 69.34 ; H, 5.20. Calcd for $C_{18}H_{16}O_5$: C, 69.22 ; H, 5.16. λ_{max}, 233 mμ (log ε, 3.99), 277 mμ (log ε, 4.11), 310 mμ (log ε, 3.84).

The 2, 4-dinitrophenylhydrazone, red prisms, m. p. 240~241°. *Anal.* Found : C, 58.81; H, 4.16; N, 11.84. Calcd for $C_{24}H_{20}O_8N_4$: C, 58.53 ; H, 4.09 ; N, 11.38.

2-Carbethoxy-7-methoxyisoflavanone (III) :

2-Carbethoxy-7-methoxyisoflavone[7], m. p. 130~131°, (3.1 g), platinum oxide (300 mg) in acetic acid (150 ml) yielded III, m.p. 95~97°, (2.6 g), which was recrystallized from ethanol to give colorless rhombs, m.p. 100~101°. *Anal* Found : C, 70.17 ; H, 5.55. Calcd for

* If not described, the hydrazones were recrystallized from acetic acid.

7) W. Baker, J. Chadderton, J.Br Harborne and W.D. Ollis, *J. Chem. Soc.*, **1953**, 1852.

$C_{19}H_{18}O_5$: C, 69.92 ; H. 5.56. λ_{max}, 235 mμ (log ε, 4.03), 276 mμ (log ε, 4.13), 311 mμ (log ε 3.88).

The 2, 4-dinitrophenylhydrazone, m. p. 230.5~232°, red prisms. *Anal* Found: C, 59.12 ; H, 4.56 ; N, 10.43. Calcd for $C_{25}H_{22}O_8N_4$: C, 59.28 ; H, 4.38 ; N, 11.06.

2, 3-Diphenyl-7-methoxychromanone (IV) :

2, 3-Diphenyl-7-methoxychromon was prepared by methylation of 2, 3-diphenyl-7-hydroxychromon[6] with diazomethane. The diphenylmethoxychromon (3.3 g), platinum oxide (250 mg) in acetic acid (180 ml) gave IV (1.57 g), which was recrystallized from methanol to colorless rhombs, m.p. 128~129°.

Evaporation of the mother liquor gave the unreacted chromon (1.31 g). *Anal* Found : C, 79.96 ; H, 5.89. Calcd for $C_{22}H_{18}O_3$; C, 79.98 ; H. 5.49. λ_{max}, 278 mμ (log ε, 4.08), 311 mμ (log ε, 3.76).

The 2, 4-dinitrophenylhydrazone, m.p. 254~255°, red prisms. *Anal* Found : C, 66.07 ; H, 4.68 ; N, 10.89. Calcd for $C_{28}H_{22}O_6N_4$: C, 65.87 ; H, 4.34 ; N, 10.98.

2-Hydroxymethyl-7-methoxyisoflavanone (V) :

2-Hydroxymethyl-7-methoxyisoflavone (500 mg), platinum oxide (50 mg) in acetic acid (50 ml) yielded V, recrystallized from *n*-hexane : ethyl acetate (2:1) to give colorless plates, m.p. 156~158°, (200 mg). *Anal* Found: C, 71.52; H, 5.65. Calcd for $C_{17}H_{16}O_4$: C, 71.82; H, 5.67. λ_{max} 230 mμ (log ε, 4.07), 274 mμ (log ε, 4.17), 311 mμ (log ε, 3.86).

The 2,4-dinitrophenylhydrazone was recrystallized from aqueous ethanol, red prisms, m. p. 203~204°. *Anal* Found : C, 59.54 ; H, 4.17 ; N, 10.16. Calcd for $C_{23}H_{20}O_7N_4$: C, 59.48 ; H, 4.34 ; N, 12.06.

2-Hydroxymethyl-7, 2'-dimethoxyisoflavanone (VI) :

2-Hydroxymethyl-7, 2'-dimethoxyisoflavone (1.2 g), platinum oxide (120 mg) in acetic acid (100 ml) gave VI, m.p. 173~178° (820 mg), recrystallized from ethyl acetate to give colorless needles, m. p. 179~181°. *Anal* Found : C, 69.04 ; H, 6.00. Calcd for $C_{18}H_{18}O_5$: C, 68.78 ; H, 5.77. λ_{max}, 275 mμ (log ε, 4.17), 312 mμ (log ε, 3.83).

The 2, 4-dinitrophenylhydrazone, m. p. 249~251°, orange amorphous solid. *Anal* Found : C, 57.66 ; H, 4.36 ; N, 11.24. Calcd for $C_{24}H_{22}O_8N_4$: C, 58.30 ; H, 4.48 ; N, 11.33.

Chromano-(3', 4', 2, 3)-chromanone (VII) :

Chromenochromon (VIII) was prepared by the method reported by F.B. LaForge[3] who described the compound to be slightly yellow crystals, m.p. 135°, but after chromatographic purification on alumina the colorless needles of m.p. 132~133° was obtain. *Anal* Found: C, 76.56 ; H, 4.15. Calcd for $C_{16}H_{10}O_2$: C, 76.79 ; H, 4.03.

Chromenochromon (400 mg), platinum oxide (60 mg) in acetic (80 ml) yielded crude chromanochromanone, m.p. 110~112° (350 mg), recrystallised from aqueous ethanol (80 %) to give colorless crystals, m.p. 162~163° (80 mg). *Anal* Found : C, 75.93 ; H, 4.92. Calcd for $C_{16}H_{12}O_3$: C, 76.18 ; H, 4.80. λ_{max}, 225 mμ (log ε, 3.95), 320mμ (logε , 3.48).

Dehydrogenation of chromanochromanone :

Into the solution of chromanochromanone (150 mg) and potassium acetate (250 mg) in ethanol (10 ml), iodine (100 mg) in ethanol (5 ml) was gradually added under reflux. After refluxing for further 30 min., water was added to the solution and the precipitate was collected. Recrystallization from aqueous ethanol (80 %) gave chromenochromon, m.p. 132~133°, (80 mg).

Acknowledgement The authors wish to thank Professor S. Takei for his encouragement in the course of this work and Professor T. Mitsui for microanalyses.

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Synthesis of Four Diastereoisomers of Dihydrorotenone

(Synthesis of Rotenoids II)

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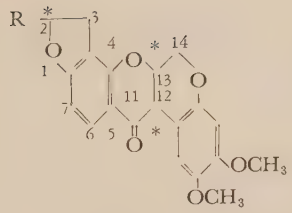
By the catalytic hydrogenation of *l*-dihydrodehydrorotenone, a stereoisomer of dihydrodesoxyrotenone and two of dihydrorotenone were prepared and termed as dihydrodesoxyallorotenone, dihydroallorotenone-I and -II respectively. Since the catalytic hydrogenation of ethylenic bonds proceeds by *cis*-addition, it seems reasonable that dihydroallorotenone-I and -II have *cis*-junction of the rings at C_{12} and C_{13}, and accordingly *l*-dihydrorotenone and *d*-dihydroepirotenone have *trans*-junction.

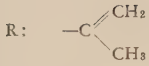
Dihydrorotenone as well as rotenone has three asymmetric carbon atoms C_2, C_{12} and C_{13}[1], which predict the presence of eight possible stereoisomers. For the dihydrorotenone which have the same configuration at the C_2 asymmetric center as that of *l*-dihydrorotenone derived from naturally occurring *l*-rotenone, four diastereoisomers can exist owing to the mode of ring junction at the two asymmetric centers, C_{12} and C_{13}. According to the Cahn's nomenclature in relation to the rotenone series, they should be designated as *l*-dihydro-, *d*-dihydroepi-, *l*-dihydroallo- and *d*-dihydroepiallo-rotenone, two of which, *l*-dihydro- and *d*-dihydroepi-isomer, have already appeared in the literature[2,3]. The preparation of the remaining two isomers are described here.

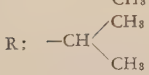
Under a similar condition described in the preceeding paper[4] *l*-dihydrodehydrorotenone[5] was hydrogenated to give rise to an oily product, which was separated into three fractions on an alumina column. The first fraction afforded colorless needles, $C_{23}H_{26}O_5$, m.p. 167~168°, which was also obtained by catalytic hydrogenation of the below mentioned dihydroallorotenone-I. The IR-spectrum of the compound informs the absence of carbonyl group, and the UV-spectrum is identical with that of *l*-dihydrodesoxyrotenone[6] prepared from *l*-rotenone by the exhaustive hydrogenation. However, the depression of the melting point of the compound was observed on admixture with *l*-dihydrodesoxyrotenone and also the value of optical rotation of these compounds differed from each other. From these facts this compound was proved to be a stereoisomer of *l*-dihydrodesoxyrotenone, being designated as *l*-dihydrodesoxyallorotenone.

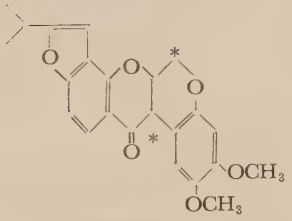
From the second and the third fraction dihydroallorotenone-I, m.p. 113~115°, and -II, m.p. 127~128°, was obtained respectively. Dihydroallorotenone-I and -II show similar IR-spectra to that of *l*-dihydrorotenone except with some differences within 1000~800 cm^{-1}. Their carbonyl groups exhibit strong absorption bands near 1675 cm^{-1}, which are displaced to higher frequency by about 36 cm^{-1} than that of *l*-dihydrodehydrorotenone. On the analogy of the case of hydrogenation of isoflavones to isoflavanones[4], these displacement of carbonyl bands

1) The numbering of carbon atoms after S. Takei, S. Miyajima and M. Ono, *Ber.*, **65**, 1041 (1932).
2) R. S. Cahn, R. F. Phipers and J. J. Boam, *J. Chem. Soc.*, **1938**, 513.
3) H. L. Haller and P. S. Schaffer, *J. Am. Chem. Soc.*, **55**, 3494 (1933).
4) H. Fukami, S. Takahashi, K. Konishi and M. Nakajima, This Bulletin, **24**, 119 (1960).
5) F. B. LaForge and L. F. Smith, *J. Am. Chem. Soc.*, **52**, 1091 (1930).
6) A. Butenandt, *Ann.*, **464**, 253 (1928).

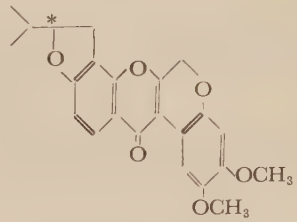


R:  Rotenone

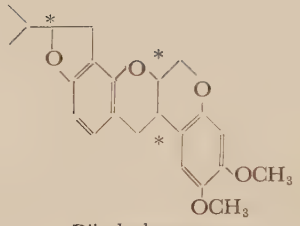
R:  Dihydrorotenone



Isorotenone



Dihydrodehydrorotenone



Dihydrodesoxyrotenone

indicate that the hydrogenation takes place on the double bond between C_{12} and C_{13} of *l*-dihydrodehydrorotenone. Moreover, the dihydrorotenone-structure of these dihydroalloisomers were also confirmed by the fact that both of them afford *l*-dihydrodehydrorotenone on the dehydrogenation with iodine under the same condition as adopted in the case of *l*-dihydrorotenone into *l*-dihydrodehydrorotenone.

On the other hand, R.S. Cahn et al.[2] obtained *d*-dihydroepirotenone, m. p. 127~128°, from gummy *d*-epirotenone by the hydrogenation of its unsaturated side chain. However, in the course of this work, *d*-epirotenone was isolated as methanol solvated crystals, m. p. 89~91°, of which hydrogenation gave *d*-dihydroepirotenone, m.p. 98°, which was dehydrogenated with iodine into *l*-dihydrodehydrorotenone. It is not yet clear whether or not our compound is identical with the compound prepared by these authors.

Values of optical rotation of thus obtained four steroisomers of dihydrorotenone and their related compounds are set in the following table.

On treatment with sulfuric acid, our crystalline *d*-epirotenone was easily converted into *d*-isorotenone, m. p. 178~179°, $[\alpha]_D$+92°, which the enantiomorph of *l*-isorotenone, m. p. 178~179°, $[\alpha]_D$−89.3°, derived from *l*-rotenone. Consequently, the mode of the ring junction at C_{12} and C_{13} of *d*-epirotenone should belong to the same series as that of *l*-rotenone. It is probable that the catalytic hydrogenation of rote-

l-Rotenone $[\alpha]_D$ −226° (Bz) → *l*-Dihydrorotenone $[\alpha]_D$ −207.7° (Bz) → *l*-Dihydrodesoxyrotenone $[\alpha]_D$ −149° (Chl)

↓ Mutarotenone $[\alpha]_D$ −84.8° (Bz)

↓ *d*-Epirotenone $[\alpha]_D$ +72.5° (Bz)

↓ *d*-Dihydroepirotenone $[\alpha]_D$ +55.7° (Bz)

l-Dihydrodehydrorotenone $[\alpha]_D$ −64° (Chl)

↓

Dihydroallorotenone-I $[\alpha]_D$ +7.4 (Bz) → *l*-Dihydrodesoxyallorotenone $[\alpha]_D$ −34° (Chl)

Dihydroallorotenone-II $[\alpha]_D$ −68° (Bz)

Bz; benzene, Chl; Chloroform

none to dihydrorotenone is only accompanied with saturation of the isopropenyl group to the isopropyl group and not having any influence on its three asymmetric centers. Therefore, *d*-dihydroepirotenone also should belong to the same series as *l*-dihydrorotenone in regard to the mode of ring junction at C_{12} and C_{13}.

In regard to the ring junction at C_{12} and C_{13} of naturally occurring *l*-rotenone, R.S. Cahn et al.[2] and more recently M. Miyano and M. Matsui[7] have proposed that the *trans*-structure appears more probable, while R. W. Hummer and E.E. Kenaga[8] inferred for *cis*-configuration. As discussed in the preceeding paper[4], it seems reasonable that dihydroallorotenone-I and -II prepared by catalytic hydrogenation have *cis*-junction of rings at C_{12} and C_{13}, and accordingly *l*-dihydrorotenone and *d*-dihydroepirotenone have *trans*-junction. Further studies are necessary to determine which of the two allo-isomers corresponds to *d*-dihydroepiallorotenone.

EXPERIMENTAL*

Catalytic hydrogenation of *l*-dihydrodehydrorotenone—One gram of *l*-dihydrodehydrorotenone was suspended in 200 ml of acetic acid, and hydrogenated in the presence of 70 mg of platinum oxide. Hydrogenation was discontinued after the absorption of 135 ml of hydrogen (2.3 moles). The catalyst was filtered and the solvent distilled off in vaccum. The residue was dissolved in chloroform, the chloroform layer was washed with 5% aqueous potassium hydroxide and with water. The chloroform solution was dried over anhydrous sodium sulfate and evaporated to a yellow syrup (ca. 1 g), which was dissolved in 5 ml of benzene and chromatographed on 20 g of alumina. The elution was divided into three fractions. The first fraction was collected by elution with 20 ml of *n*-hexane and followed by 30 ml of benzene-*n*-hexane (3 : 2). The second and third fractions were collected by elution with each 40 ml of benzene.

Evaporation of the first fraction gave 100 mg of dihydrodesoxyallorotenone. After recrystallization from ethanol the compound melted at 167～168°. The melting point of the compound was depressed on admixture with *l*-dihydrodesoxyrotenone prepared from *l*-rotenone (m.p. 170～171°, $[\alpha]_D$ −149° chloroform), λ_{max} 290 mμ, (log ε, 3.87)). *Anal.* Found : C, 72.05 ; H, 7.05. Calcd. for $C_{23}H_{26}O_5$: C, 72.23 ; H, 6.85. λ_{max} 290 mμ (log ε 3.87), λ_{min} 258 mμ (log ε 2.94). $[\alpha]_D$ −34° (chloroform).

The second fraction afforded 300 mg of colorless needles. Recrystallization from methanol gave MeOH-solvate of dihydroallorotenone-I, m.p. 103～105°. *Anal.* Found : C, 68.20 ; H, 6.27. Calcd. for $2C_{23}H_{26}O_6 \cdot CH_3OH$: C, 68.45 ; H, 6.31. The methanol-solvate was heated for 7 min. at 125° and then recrystallized from *n*-hexane, non-solvated colorless prisms, m.p. 113～115° were obtained. *Anal.* Found : C, 69.86 ; H, 6.27. Calcd. for $C_{23}H_{24}O_6$: C, 69.68 ; H, 6.10. λ_{max} 238 mμ (log ε 4.08), 295 mμ (log ε 4.14) ; λ_{min} 233 mμ (log ε 4.04), 260 mμ (log ε 3.50). $\lambda_{C=O}$ 1672 cm^{-1}. $[\alpha]_D$ +7.4 (benzene). The hydrogenation of the compound in acetic acid over platinum oxide also gave dihydrodesoxyallorotenone as described above.

The third fraction gave 50 mg of dihydroallorotenone-II, colorless needles, m.p. 123～125°. After recrystallization from methanol the compound melted at 127～128°. *Anal.* Found : C, 69.71 ; H, 6.08. Calcd. for $C_{23}H_{24}O_6$: C, 69.68 ; H, 6.10. λ_{max} 238 mμ (log ε 4.08), 295 mμ (log ε 4.14) ; λ_{min} 233 mμ (log ε 4.04), 260 mμ (log ε 3.48). $\lambda_{C=O}$ 1675 cm^{-1}. $[\alpha]_D$ −68° (benzene).

***d*-Epirotenone**—When mutarotenone[2] (1.4 g) was dissolved in carbon tetrachloride, 0.8 g of *l*-rotenone-CCl_4 solvate was separated. On evaporation of the mother liquor gummy *d*-epirotenone (1.0 g) was obtained and dissolved in ether. After being allowed to stand in a refrigerator, small amounts of mutarotenone were separated and the filtrate was first washed with aqueous 1 N-NaOH, followed by water and dried over anhydrous sodium sulfate. The ether was distilled off and the residue was dissolved in methanol. After being kept standing for a prolonged period, *d*-epirotenone-methanol solvate (380 mg) slowly separated, m.p. 89～91°. *Anal.* Found : C, 68.58 ; H, 5.95. Calcd. for $2C_{23}H_{24}O_6 \cdot CH_3OH$: C, 68.77 ; H, 5.89. $[\alpha]_D$ +72.5 (benzene).

***d*-Dihydroepirotenone**—Seven hundred milligrams of *d*-epirotenone methanol solvate in 40 ml of ethyl acetate was hydrogenated in the presence of platinum oxide until 46 ml of hydrogene was absorbed. The catalyst was filtered and the filterate was evaporated to dryness. Recrystallization from ethanol gave 580 mg of colorless needles, m. p. 104～106°. *Anal.* Found : C, 68.96 ; H, 6.41. Calcd. for $2C_{23}H_{24}O_6 \cdot C_2H_5OH$: C, 68.72, H, 6.49. $[\alpha]_D$ +55.8° (benzene).

7) M. Miyano and M. Matsui, *Chem. Ber.*, **91**, 2044 (1958).
8) R. W. Hummer and E. E. Kenaga, *Science*, **113**, 653 (1951).
* All melting points are uncorrected. Optical rotation was measured at room temperature (13～20°). IR-spectra were measured in nujiol, UV-spectra in ethanol solution.

Heating at 80~83° for 12 hr. in vacuum afforded non-solvated crystals of m. p. 98°. *Anal.* Found: C, 69.62, H, 6.12. Calcd. for $C_{23}H_{24}O_6$: C, 69.68, H, 6.10. λ_{max} 238 mμ (log ε 4.10), 295 mμ (log ε 4.14), λ_{min} 233 mμ (log ε 4.04), 260 mμ (log ε 3.50). $\lambda_{C=O}$ 1664 cm^{-1}, $[\alpha]_D$ +51.4° (benzene).

Dehydrogenation of dihydrorotenone isomers—Fifty milligrams of dihydroallorotenone-I and 100 mg of potassium acetate were dissolved in 20 ml of alcohol and 50 mg of iodine in alcohol was added in small portions. On cooling, *l*-dihydrodehydrorotenone (20 mg) was separated out, collected and washed with water. This substance was recrystallized from alcohol, m. p. 222~223°. By application of the same method, dihydroallorotenone-II and *d*-dihydroepirotenone also gave *l*-dihydrodehydrorotenone.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2, p. 126~130, 1960].

Studies on Osmophilic Yeasts

Part VII. Production of Polyalcohols by *Saccharomyces rouxii* in the Concentrated Media of Sodium Chloride and Sugars, and Identification of Polyalcohols Produced

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Identification of polyalcohols which were produced in large amounts by *Saccharomyces rouxii* under aerobic fermentation in the concentrated media of sodium chloride and sugar, was carried out by isolating these products in crystalline form from the fermented broth. The products were determined as glycerol and D-arabitol. In the concentrated medium of sodium chloride, about 70% of the polyalcohols produced was glycerol, while in the concentrated sugar medium, the formation of D-arabitol was more markable than glycerol.

INTRODUCTION

In the previous paper[1], the author reported that *Saccharomyces rouxii*, which is a typical salt-tolerant yeast and plays an important role in soy-brewing, is found to ferment glucose giving high yields of glycerol in the high saline medium under aerobic conditions, though it produces a small amount of glycerol in the ordinary medium. From the results of paper chromatography and partition chromatography using celite, it was also reported that the majority of values obtained as crude glycerol is found to be true glycerol, a small amount of polyalcohol other than glycerol being detected.

Spencer and Sallans[2] found that osmophilic *Zygosaccharomyces* isolated from fermenting honey and other sources produces one or more of the following sugar alcohols, glycerol, erythritol, D-arabitol, mannitol and two unidentified polyhydric alcohols under aerobic conditions, and carried out detailed studies on the factors

1) H. Onishi, This Bulletin, **23**, 359 (1959)

2) J. F. T. Spencer and H. R. Sallans, *Can. J. Microbiol.*, **2**, 72 (1956).

favoring glycerol production[3]. Peterson et al.[4] also investigated factors affecting production of glycerol and D-arabitol employing representative yeasts of the genus *Zygosaccharomyces*.

It is noted that all of these studies are carried out in the ordinary medium and not in the concentrated saline medium. In the case of *Sacch. rouxii*, a salt-tolerant soy yeast, effect of NaCl concentrations of the medium on the yields of glycerol was very remarkable as previously stated[1]. For the purpose of identification of polyacohols produced by aerobic fermentation in the two concentrated media of glucose and sodium chloride, isolation in crystalline form from the fermented broths was carried out, from which they were determined as glycerol and D-arabitol. In the concentrated salt medium, about 70% of polyalcohols produced were glycerol, while in the concentrated glucose medium, a large amount of D-arabitol was produced than in case of glycerol.

EXPERIMENTAL AND RESULTS

[I] Cultural condition.

Yeast strain employed: *Sacch. rouxii* m 3[1]

The two media used were as follows: (1) the concentrated saline medium: glucose 10%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 18.0%, casamino acid 0.4%, yeast-ext. 0.1%, pH 4.4, (2) the concentrated glucose medium: glucose 30%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%, casamino acid 0.4%, yeast-ext. 0.1%, pH 4.8.

Eighty ml of these media were placed in a 500-ml shake-flask, sterilized and then one drop of fresh culture suspension was inoculated. The flasks were shaken on a reciprocal shaker operating at 120 r.p.m. with a stroke of 75 mm at 30°. After 9 days' incubation, the fermentation products were subjected to analysis.

[II] Analysis of the fermented broth.

The cleared fermentation solutions from which the yeast cells were removed by filtration were analysed according to the following methods.

Glucose: The Fehling-Lehmann-Schoorl method[5].

Ethanol: After distillation in neutral (pH 7.6~7.8), the oxidation method by dichromate was applied.

pH: pH test paper (Toyo Roshi Kaisha)

Total acid: Ether extracts in acid reaction with H_2SO_4, are titrated with N/20 NaOH.

Total polyalcohols (calculated as glycerol): the method of Desnuelle and Naudet[6] as described by Neish[7].

Total polyalcohols were determined by oxidation with periodic acid followed by colorimetric determination of formaldehyde formed with a chromotropic acid reagent. The values obtained were corrected for glucose presented by running a standard glucose sample in parallel with the fermentation solutions.

True glycerol: Glycerol was separated on water-celite columns using a mixture of 25% benzene and 75% *n*-butanol saturated with water as a developing solvent and analysed by the above periodic oxidation procedure. The results are shown in Table I.

Table I. Analytical Results of the Fermented Broth

	18% NaCl medium*1)	30% glucose medium*2)
% of glucose consumed	9.16	23.04
rate of glucose consumption (%)	99.2	80.3
% of ethanol formed	0.09	2.09
rate of alcohol fermentation on glucose consumed	1.92	17.74
rate of other consumption on glucose consumed	98.08	82.26
total acid formed (%)	0.12	0.17
% of crude glycerol formed	3.938	6.303
yield of crude glycerol on glucose consumed	43.0%	27.4%
% of true glycerol formed	2.925	3.150
yield of true glycerol on glucose consumed	31.9%	13.7%
ratio of true glycerol to crude glycerol	0.75	0.50

*1) Initial glucose concentration is 9.23% and initial pH 4.4.
*2) Initial glucose concentration is 28.69% and initial pH 4.8.

[III] Isolation of each polyalcohol from the fermented broths.

As shown in Table I, 75% of total polyalcohols (calculated as glycerol) produced in the concentrated saline medium were true glycerol, while in the concentrated glucose medium, polyalcohol other than glycerol was formed in larger quantities than glycerol: that is to say,

3) J. F. T. Spencer, J. M. Roxburgh and H. R. Sallans, *J. Agr. Food Chem.*, **5**, 64 (1957).
4) W. H. Peterson, W. F. Hendershot and G. J. Hajny, *App. Microbiol.*, **6**, 349 (1958).
5) A. W. Van der Haar, Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und Aldehydsäuren, Berlin (1920).
6) P. Desnuelle and M. Naudet, *Bull. Soc. Chim. France*, **12**, 871 (1945).
7) A. C. Neish, Analytical method for bacterial fermentation. 2 nd rev. Natl. Research Council. Can., Praire Regional Lab., Saskatoon, Saskatchewan N. R. C. No. 2952 (1952).

the ratio of glycerol to other polyalcohol products differed in the two concentrated media. In order to find whether or not the same polyalcohols are formed in these two concentrated media, the isolation and crystallization of each polyalcohol from the fermented broth were conducted as follows.

Residual glucose, which interferes with isolation of polyalcohols from the fermented broth, was removed by fermentation with baker's yeast. In this case, it was also confirmed that baker's yeast could only produce glycerol and not other polyalcohols; when the 10% glucose medium was fermented by baker's yeasts, other polyalcohols except glycerol could not be detected on the paper chromatogram of the fermented broth. The method of paper chromatography was as follows: it was developed with the solvents composed of *n*-butanol, ethanol and water (4 : 1 : 5 by volume[8]) and of *n*-propanol, ethylacetate and water (7 : 1 : 2 by volume[9]), and the polyalcohols were detected by the method of Yoda[10].

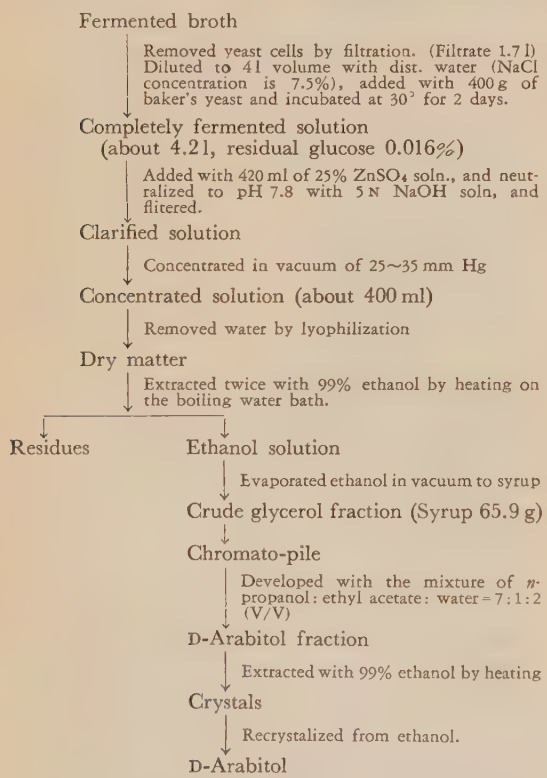


FIG. 1. Isolation and Purification of Each Polyalcohol from the Fermented Broth of the Concentrated Sodium Chloride.

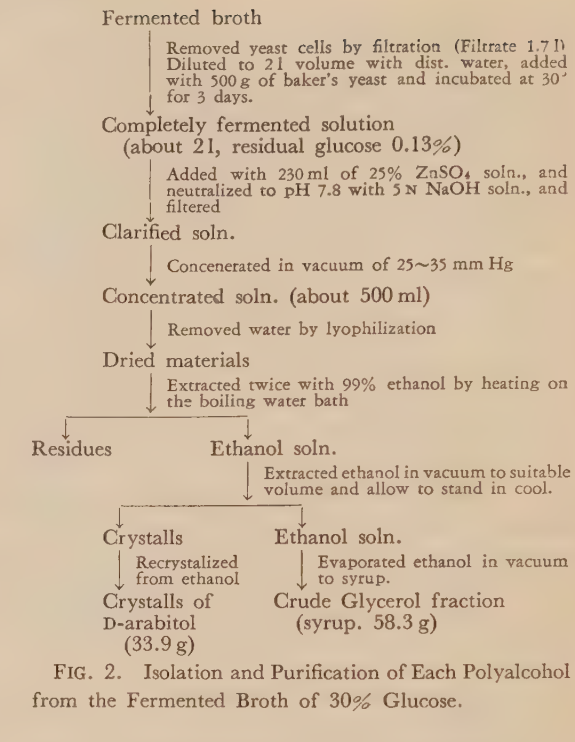


FIG. 2. Isolation and Purification of Each Polyalcohol from the Fermented Broth of 30% Glucose.

The fermented solution which was made almost completely free from glucose, was clarified by the addition of a 1/10 volume of 25% zinc sulfate solution to the fermented solution and adjusting the pH value to 7.8 with 5 N NaOH solution, followed by filtration.

This clarified solution was concentrated by vacuum evaporation of 25~35 mmHg at 45~50°, and further completely dehydrated by lyophilization. The dried material was extracted twice with 99% ethanol by heating on a boiling water bath, and from this ethanol solution, D-arabitol was crystallized.

Procedures of isolation of glycerol and D-arabitol from the two concentrated media of sodium chloride and glucose are illustrated in Figs. 1 and 2.

By the above procedures, D-arabitol was only obtained in crystalline form from the concentrated glucose medium, but crystallization from the concentrated sodium chloride medium was not successful though the ethanol solution was concentrated to syrup. Then, it was shown by paper chromatography that this syrup contains both

8) R. H. Hackman and V. M. Trikojus, *Bicohem. J.*, **51**, 653 (1952).

9) J. Cerbulis, *Anal. Chem.*, **27**, 1400 (1955).

10) A. Yoda, *J. Chem. Soc. Japan*, **73**, 18 (1952).

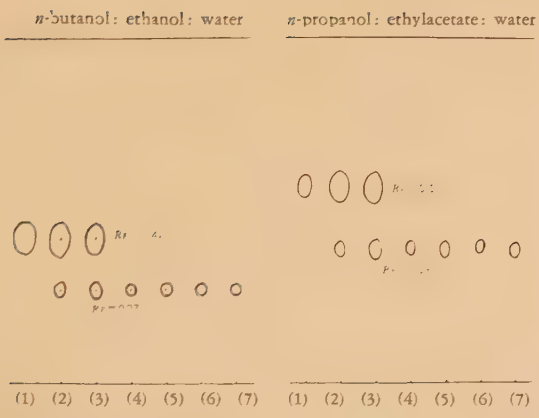
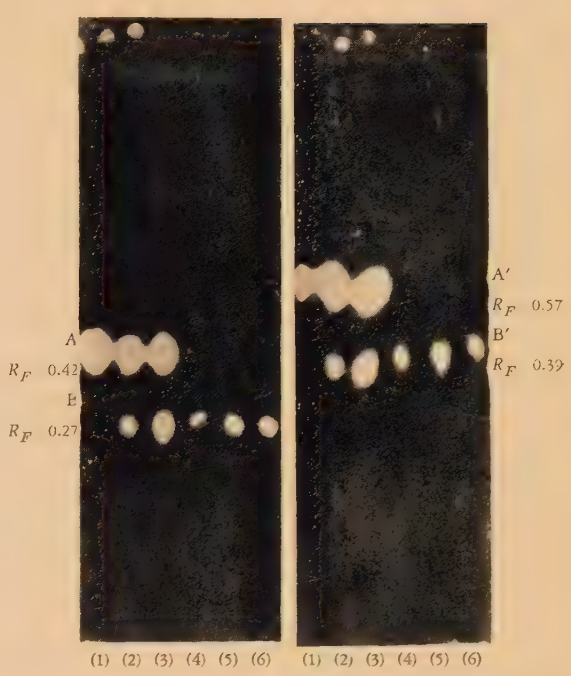


FIG. 3. Paper Chromatograms of Polyalcohols Isolated.

1) Authentic glycerol.
2) Crude glycerol fraction from 18% NaCl medium.
3) Crude glycerol fraction from 30% glucose medium.
4) Authentic adonitol.
5) Crystals obtained from 30% glucose medium,
6) Authentic D-arabitol.
7) Crystals obtained from 18% NaCl medium.

glycerol and D-arabitol (see Fig. 3 and Photograph 1). In this case, it seems that D-arabitol is dissolved in glycerol which is produced in a larger amount than D-arabitol. The application of chromatopile method was successful in the separation of these two polyalcohols each other. A stack of 800 sheets of filter paper discs was clamped together, and developed with the solvent composed of *n*-propanol, ethylacetate and water (7 : 1 : 2 by volume). Then, the discs were separated and the



Photograph 1. Paperchromatograms of Polyalcohol Isolated.

1) Authentic glycerol.
2) Crude glycerol fraction from 18% NaCl medium.
3) Crude glycerol fraction from 30% glucose medium.
4) Authentic adonitol.
5) Crystals obtained from 30% glucose medium.
6) Authentic D-arabitol.

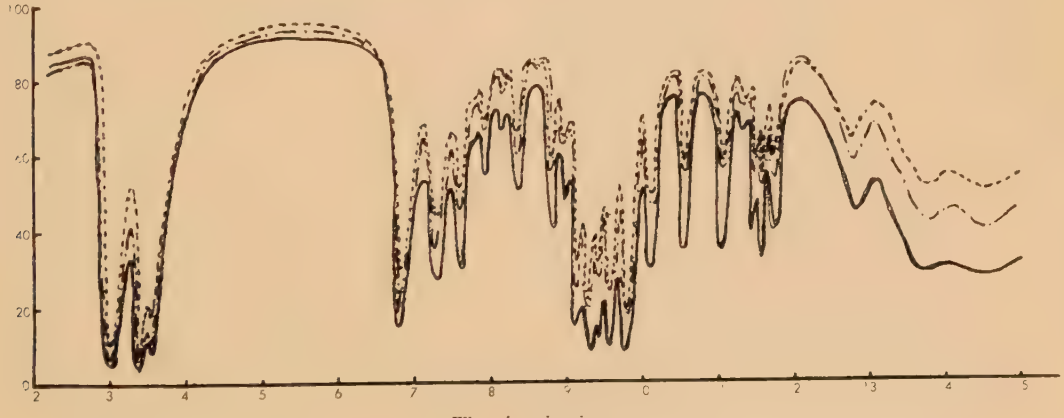


FIG. 4. Infra-red Spectra of D-Arabitol.

——: Crystals obtained from 18% NaCl medium.
- - - -: Crystals obtained from 30% glucose medium.
— - —: Authentic D-arabitol.

constituents were tested for (R_F values were almost the same as those in paper chromatography). The discs of D-arabitol fraction were extracted with 99% ethanol by heating on a boiling water bath, and crystallization of D-arabitol from the ethanol solution was accomplished.

[IV] Identification of glycerol.

(1) Preparation of glycerol tribenzoate[11]: Place 100 mg of glycerol in a 25×200 mm test tube and add 0.5 ml of benzoyl chloride. Select a solid rubber stopper that securely fits the mouth of the tube. Add 5 ml of 10% sodium hydroxide solution and shake vigorously until the solid derivative separates out. Allow to stand in a cold bath, shaking the tube at intervals so that the lumps forming at the beginning break up into small granules; use a rod for this purpose if necessary. Add 5 ml of water, shake vigorously for a few minutes, and then filter. Wash twice with 5 ml of water and place on a drying disc. Glycerol tribenzoate is recrystallized from ligroins as needles, m.p. 71°. *Anal* Found: C, 71.18%; H, 5.47%. Calcd. for $C_{24}H_{22}O_6$: C, 71.27%; H, 4.99%.

This crystal did not depress the melting point on admixture with an authentic specimen which was prepared from special grade glycerol.

(2) Paper chromatography: As shown in Fig. 3 and Photograph 1, the spots of both glycerol fractions from the media of 18% NaCl and 30% glucose, agreed with those of the authentic glycerol.

[V] Identification of D-arabitol.

Three recrystallizations from ethanol, afforded prismatic crystals melting at 101°. This did not depress the melting point on admixture with an authentic specimen.

Two crystals from the media of 18% NaCl and 30% glucose, showed similar analytical results.

$[\alpha]_D^{20}=+8.28$ (C=9.78, in saturated borax solns.)
Anal. Found: C, 39.40%; H, 7.98% (from 30% glucose medium); C, 40.48%; H, 7.90% (from 18% NaCl medium). Calcd. for $C_5H_{12}O_5$: C, 39.41%; H, 7.89%.

The infrared spectra of these samples were also found to be superimposable with that of the authentic specimen (Fig. 4).

As illustrated in Fig. 3, spots of these crystals agreed with those of the authentic D-arabitol.

Acknowledgement The author wishes to express his sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragement throughout this work. Great indebtness is also acknowledged to Prof. T. Asai, Prof. Y. Sumiki, Prof. K. Kitahara, Prof. K. Arima and Prof. Y. Ikeda for their valuable suggestions. Thanks are also due to Prof. Y. Sumiki for the precious gift of D-arabitol, and to the Department of Agricultural Chemistry, University of Tokyo, for the infrared spectroanalysis and the microanalysis. The author also wishes to thank Dr. M. Mogi, the Director of Noda Institute for Scientific Research, for his encouragement and Mr. N. Saito for his willing help throughout the course of this work.

11) N. D. Cheronis and J. B. Entriken, Semimicro qualitative organic analysis, 2nd Ed., p. 383 (1957).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2, p. 131~140, 1960]

Studies on Osmophilic Yeasts

Part VIII. Polyalcohol Production by Various Genera and Species of Yeasts

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From the results of investigation of the polyalcohol productivity of various genera and species of yeasts, the present author has now found that all the yeast strains tested show different individual characteristics in polyalcohol productivities and can be classified in the following six types: (1) solely producing glycerol, (2) solely producing erythritol, (3) producing only D-arabitol, (4) producing erythritol and D-arabitol, (5) producing glycerol and D-arabitol, and (6) producing glycerol, erythritol and D-arabitol.

It was also found that *Pichia miso*, which was isolated from miso-paste and showed a high osmophilic property, could produce glycerol, erythritol and D-arabitol giving very high yields, 50~60%, of sugar consumed. It seems to be worthy of note that this yeast was able to completely dissimilate highly concentrated glucose, such as 30%, producing much polyalcohols as those state above.

INTRODUCTION

In the previous paper[1,2], the author has found that *Sacch. rouxii*, which is a typical salt-tolerant yeast and plays an important role in soy-brewing, produces polyalcohols redering high yields under aerobic fermentation in the concentrated media of NaCl or sugar, and the polyalcohols produced were identified as glycerol and D-arabitol by isolation in crystalline form from the fermented broths.

Studies on the production of polyalcohols other than glycerol by yeasts have not yet been reported with the exception of the production by genus *Zygosaccharomyces* alone, which has been shown by Spencer and Sallans[3], and Peterson et al.[4]. Previous knowledges are not available for the correlation between the genera and species of yeasts and their polyalcohol productivities.

Here-upon, the author has conducted extensive studies on polyalcohol production by various genera and species of yeasts including many osmophilic yeasts, from which it was revealed that these yeasts are classified into six types according to their characteristic productivities of the three polyalcohols, glycerol, erythritol and D-arabitol. Also, it was found that *Pichia miso*, which was isolated from miso-pastes by Mogi[5], can dissimilate sugar almost complety in a concentrated glucose solution such as 30%, producing glycerol, erythritol and D-arbitol with a high yield of 50~60% of glucose consumed.

EXPERIMENTAL AND RESULTS

[I] Polyalcohol production by various genera and species of yeasts.

Cultural conditions and analytical methods:

Since all of the 156 strains tested were able to grow well in a 30% glucose medium, polyalcohol production was examined by employing concentrated glucose medium composed of the following constituents: glucose 30%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot$

1) H. Ōnishi, This Bulletin, **23**, 359 (1959).
2) H. Ōnishi, This Bulletin, **24**, 126 (1960).
3) J. F. T. Spencer and H. R. Sallans, *Can. J. Microbiol.*, **2**, 72 (1956).
4) W. H. Peterson, W. F. Hendershot and G. J. Hajny, *App. Microbiol.*, **6**, 349 (1958).
5) M. Mogi, *J. Agr. Chem. Soc. Japan*, **15**, 921, 1023, 1221 (1939), **16**, 7 (1940).

Table I. Production of Polyalcohols by Various Genera and Species of Yeasts.
(1) *Saccharomyces*.

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohols produced		
				D-arabitol	erythritol	glycerol
S. aggultianus Forma I	13.0	0.73	5.6%	−	−	++
S. aggultianus Forma II	8.7	0.64	7.3%	−	−	++
S. awamori	7.2	0.63	8.7%	−	−	++
S. anamensis	13.0	0.73	5.6%	−	−	++
S. cartilaginosus	2.0	0.31	15.5%	−	−	+
S. chevalieri	8.5	0.90	10.5%	−	−	++
S. exiguus	2.3	0.32	13.9%	−	−	+
S. fragilis	20.7	2.22	10.7%	++	−	+++
S. intermedius	4.0	0.45	11.2%	−	−	+
S. lindneri	10.1	0.78	7.7%	−	−	++
S. logos	7.5	0.27	3.6%	+	−	−
S. monasensis	4.6	0.68	14.7%	−	−	++
S. mongolicus	5.0	0.86	17.2%	−	−	++
S. paradoxus	9.7	0.94	9.6%	−	−	++
S. validus	2.8	0.37	13.2%	−	−	+
S. wilianus	8.0	0.78	9.7%	−	−	++
S. marxianus	11.1	1.59	14.3%	+	−	++
S. unisporus	2.4	0.52	21.6%	−	−	+
S. pasteurianus	3.3	0.47	14.2%	−	−	+
S. saké Kyokai 6	12.7	1.09	8.5%	−	−	++
S. carlsbergensis	7.9	0.70	8.8%	−	−	++
S. dairensis	5.2	0.46	8.8%	−	−	+
S. acidifaciens	19.7	5.13	26.1%	+++	−	+++
S. acidifaciens var. *halomembranis*	21.2	4.90	23.1%	+++	−	+++
S. manginii var. *miso*	9.3	0.82	8.8%	−	−	++

$2H_2O$ 0.01%, Casamino acid 0.4%, Yeast extract 0.1%, pH 4.8.

One drop of freshly cultured yeast suspension was inoculated into 500-ml shake-flasks containing 80 ml of the medium. The flasks were shaken on a reciprocal shaker operating at 120 r.p.m. with a stroke of 75 mm at 30°. After 10 days' incubation, the fermentation products were analysed according to the method of Neish[6]. At the same time, the fermented broth was subjected to paper chromatography to determine qualitatively the polyalcohol produced. Paper chromatograms were developed with a solvent composed of *n*-propanol, ethylacetate and water (7 : 1 : 2 by volume[7]) and the polyalcohols were detected according to the method of Yoda[8].

The results are shown in Table I.

As the results of this examination, some characteristic differences in polyalcohol production could be recognized among various genera of yeasts; genus *Saccharomyces* which has been known as fermenting type, produced a relatively small quantity of polyalcohol, which mostly consisted of glycerol alone. (Typical strain : *Saccharomyces saké* and *Saccharomyces carlsbergensis*). In genus *Zygosaccharomyces*, a larger amount of polyalcohols was formed as compared with genus *Sacchromyces* and most of polyalcohols formed consisted of glycerol and D-arabitol. (Typical strains : *Saccharomyces rouxii* (*Zigosaccharomyces major*[1,2]))

In genus *Debaryomyces*, which is a no fermentation or a very weak fermentation type, almost all of the strains produced only D-arabitol (Typical strain : *Debaryomyces*

6) A.C. Neish, Analytical method for bacterial fermentation 2 nd Rev., Natl. Research Council Can., Prairie Regional Lab., Saskatoon, Sask. N. R. C. No. 2952 (1952).

7) J. Cerbulis, *Anal. Chem.*, **27**, 1400 (1955).

8) A. Yoda, *J. Chem. Soc. Japan*, **73**, 18 (1952).

(2) *Zygosaccharomyces*.

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
Z. bisporus	20.8	0.95	4.5%	++	−	++
Z. dairensis	12.1	0.62	5.1%	−	−	++
Z. fermentati	8.6	0.30	3.5%	−	−	−
Z. mellis-acidi	8.8	1.59	18.1%	++	−	++
Z. nadsonii	2.7	1.05	38.8%	++	−	++
Z. nukamiso	15.0	5.10	34.0%	++	−	++
Z. naniwaensis	20.3	1.33	6.5%	++	−	++
Z. vini	19.3	3.43	17.7%	++	−	++
Z. miso	20.3	2.16	10.5%	++	−	++
Z. amoebroidieus	12.2	1.93	15.9%	++	−	++
Z. citrus	14.2	3.45	24.3%	++	−	++
Z. felsineus	15.3	2.02	13.2%	++	−	++
Z. gracilis	13.7	3.03	22.1%	++	−	++
Z. gracilis var. *italicus*	21.2	3.40	15.9%	++	−	++
Z. mellis	7.3	2.15	29.4%	++	−	++
Z. nussbaumeri	2.9	1.12	38.6%	++	−	±
Z. polymorphus	11.0	2.29	20.8%	++	−	++
Z. rugosus	16.2	1.27	7.8%	++	−	±
Z. variabilis	17.9	2.09	11.7%	++	−	++
Z. halomembranis 433	5.7	0.95	16.7%	−	−	++
Z. halomembranis 471	18.6	3.65	19.0%	++	−	++

(3) *Hansenula*

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
H. saturnus	14.9	0.77	5.2%	++	−	−
H. suaveolens	1.6	0.14	8.7%	−	−	−
H. anomala	6.2	1.31	21.1%	±	−	+
H. anomala var. *productiva*	7.2	1.65	22.9%	+	−	+
H. mrakii	1.3	0.42	32.3%	−	−	−
H. schneggii	3.6	0.94	26.1%	+	−	+
H. miso β	20.5	4.94	24.8%	++	−	++
H. anomala var. *miso*	8.2	1.20	14.6%	++	−	++
H. miso β var. 1	9.9	2.38	24.0%	++	−	++
H. miso α	18.0	0.95	5.3%	+	−	++
Pseudohansenula peiping	16.7	1.94	9.7%	++	−	++

saké). Genus *Hansenula* formed one or two of D-arabitol and/or glycerol, and genus *Pichia* which, in a general, was an excellent producer of polyalcohols, produced two or three of glycerol, D-arabitol and erythritol. Especially, *Pichia miso*[5] was found to be the most superior strain for polyalcohol production among the 156 strains tested, giving a markedly high yield as 12.24 g of polyalcohol (calculated as glycerol) was formed from 28.6 g glucose dissimilated. *Pichia miso* which was isolated from miso-pastes and originally named by Mogi[5], was

(4) *Debaryomyces*

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
D. fabryi	10.5	0.22	2.1%	+	−	−
D. gruetzii	9.0	0.48	5.3%	++	−	−
D. hunderoi	9.6	0.49	5.1%	++	−	−
D. kloeckeri 7570	17.3	0.81	4.7%	++	−	−
D. kloeckeri 7050	8.1	0.29	3.5%	+	−	−
D. mandschuricus	10.1	0.35	3.4%	−	−	−
D. membranaefaciens	19.3	5.05	26.2%	+++	−	+++
D. membranaefaciens var. *Zingiberi*	7.7	0.85	11.1%	+++	−	−
D. saké	10.0	2.05	20.5%	+++	−	−
D. saké Froma I	9.0	0.48	5.3%	++	−	−
D. tyrocola	10.4	0.35	3.3%	++	−	−
D. tyrocola α	6.9	0.42	6.1%	++	−	−
D. tyrocola strain Okunuki	10.4	0.35	3.3%	++	−	−
D. kloeckri	6.4	0.66	10.3%	++	−	−
D. miso var. 1	8.4	1.84	21.9%	+++	−	−
D. matruchoti	9.0	0.62	6.8%	+	−	−

(5) *Pichia*

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
P. alcoholophila	23.1	5.43	23.5%	+++	−	+++
P. belgica	12.1	3.11	25.7%	+++	−	+++
P. farinosa	15.3	1.87	12.2%	+++	−	+++
P. hyalospora	16.6	1.74	10.4%	+++	±	++
P. mandschurica (1)	16.3	1.90	11.7%	+++	±	++
P. mandschurica (2)	16.1	2.21	13.7%	++	−	++
P. membranaefaciens	22.0	4.65	21.1%	+++	−	+++
P. polymorpha	21.0	4.72	22.4%	+++	−	+++
P. quercibus	8.9	0.18	2.0%	−	−	−
P. farinosa	14.3	1.22	8.5%	++	−	+
P. miso 7050	29.4	10.76	36.5%	+++	+	+++
P. miso 7061	28.1	9.11	32.4%	+++	−	+++
P. farinosa A 6	18.0	4.19	23.2%	+++	−	+++
P. miso H 52	28.6	12.24	42.4%	+++	++	+++
Zygopichia miso	17.0	1.28	7.5%	+++	++	+++
P. membranaefaciens	13.1	2.40	18.3%	++	−	+++

rena med as *Pichia mogii*[9] or as *Pichia saké* form α[10] by subsequent investigators. In the anascosporogenous yeasts, various types of polyalcohol production were observed in strain by strain. Among those, *Torulopsis famata*[11] and *Candida polymorpha*[11], both of which were isolated from soy-mashes at the initial stage of brewing and showed strong salt-tolerant and non-fermenting property,

9) I. Ohara and H. Nonomura, *J. Agr. Chem. Soc. Japan*, **28**, 122 (1954).

10) K. Kodama, *J. Fermentation Technol.*, **33**. 455 (1955).

11) H. Ōnishi, This Bulletin, in press.

(6) Other genera of yeasts

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
Candida tropicalis	15.8	1.76	11.1%	++	−	+
C. lipolytica	5.2	0.36	6.9%	−	−	−
C. guilliermondii	9.5	1.40	14.7%	+	−	+
C. parapsilosis	11.9	1.23	10.3%	++	−	+
C. krusei	27.7	4.48	16.2%	++	−	+++
C. tropicalis A	20.3	1.48	7.2%	+++	−	+
C. tropicalis SA	12.1	0.91	7.5%	+++	−	−
C. arborea	6.1	0.70	11.5%	+++	−	−
Torulopsis halophilus	8.0	0.19	2.4%	−	−	−
Torulopsis nodaensis	8.2	0.42	5.1%	−	−	+
T. spherica	14.2	5.17	36.4%	−	−	+++
T. varsatilis	13.9	6.81	48.8%	−	−	+++
T. osloensis	18.3	2.82	24.9%	+++	−	+++
T. rubra	8.1	1.83	22.5%	−	−	++
T. uvae var. *miso*	7.2	0.73	10.1%	++	−	−
T. etchellsii	6.8	0	0	−	−	−
T. famata	13.7	4.11	30.6%	+++	++	−
C. polymorpha	7.5	1.67	22.2%	+++	++	−
Kloeckera africana	2.0	0.39	19.5%	−	−	+
Rhodotorula rosa	4.0	0.88	22.0%	++	−	+
Pseudomycoderma miso α	22.5	5.26	23.3%	+	−	+++
Pseudomycoderma miso β	6.5	1.96	30.1%	+++	−	+++
Monilia formosa	4.8	1.29	26.9%	+++	−	+++
Monilia vini	9.9	1.39	14.0%	+++	−	++
Monilia sitophila	16.8	1.90	11.3%	+++	−	++
Monilia sp.	5.1	1.56	30.5%	+++	−	+++
Mycotorula japonica	18.2	1.53	8.3%	+++	−	++
Mycoderma cerevisiae	2.3	0.27	11.6%	−	−	−
Geotricum candidum	2.1	0.62	29.5%	−	−	+
Trigonopsis variabilis	8.0	0.78	9.7%	−	++	−

(7) Miscellaneous yeasts

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
S 3	28.9	9.28	32.1%	+++	−	+++
S 6	6.4	0.89	12.3%	++	−	−
S 13	18.7	2.53	13.6%	+++	−	+
S 14	10.1	0.40	4.0%	++	−	−
S 15	6.9	0.14	2.0%	+	−	−
m 12	19.8	2.81	14.2%	+++	−	+++
m 13	12.2	1.00	8.2%	+++	−	−
7484	9.3	1.02	10.9%	−	−	++
E 1	20.2	2.73	13.5%	++	−	++
E 3a	23.1	2.85	12.3%	+++	+	++
E 3b	19.2	3.91	20.3%	+++	+	++
E 3c	24.5	2.12	8.6%	+++	−	++
E 10	18.1	2.15	11.8%	++	−	++
E 11a	5.7	1.04	18.2%	+	−	+
E 11c	19.1	2.95	15.4%	++	+	+++
E g2	5.8	0.97	16.7%	−	−	+
E g3	19.0	1.83	9.5%	+++	−	+
E h	6.2	1.13	18.2%	++	−	+

Table II. Effect of Glucose Concentration in the Medium on Polyalcohol Production by *Pichia miso*.

glucose concentration of medium	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
9.93%	9.82	1.84	18.7%	###	−	±
19.78%	19.19	6.13	31.9%	###	−	##
29.24%	27.65	11.91	43.1%	###	##	##
40.73%	23.15	9.80	42.3%	###	##	##
47.94%	15.52	5.20	33.5%	##	±	##

Table III. Effect of Nitrogen Sources on Polyalcohol Production by *Pichia miso*.

nitrogen source and its concentration	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
beef extract 0.4%	11.88	4.64	39.1%	##	±	##
corn steep liquor 0.4%	20.24	8.78	43.3%	##	##	##
polypeptone 0.4%	18.24	9.98	54.7%	###	##	###
yeast extract 0.4%	28.80	11.19	38.8%	###	##	###
ammonium lactate 0.2%	28.85	12.09	41.9%	###	##	###
urea 0.05%	23.98	10.15	42.3%	##	##	###
ammonium sulfate 0.2%	12.32	2.07	16.8%	##	−	##
ammonium chloride 0.2%	11.69	1.81	15.4%	+	−	##
ammonium sulfate 0.2% added with buffer	27.54	9.46	34.3%	###	+	###
ammonium chloride 0.2% added with buffer	23.32	7.79	33.4%	###	+	###

produced both of D-arabitol and erythritol in a relatively high yield. *Trigonopsis variabilis* produced only erythritol.

As a rule, osmophilic yeasts isolated from soy-mashes and miso-pastes were found to be excellent for polyalcohol production judging from the high yield of sugar consumed and the large absolute amount produced.

[II] Effects of various cultural conditions on polyalcohol production by *Pichia miso*.

Effects of various cultural conditions on polyalcohol production were observed employing *Pichia miso* H 52 which was proved to be the most excellent strain for polyalcohol production as shown in [I]

(1) Effect of glucose concentration.

As shown in Table II, the glucose concentration of 30~40% was most preferable for the production of polyalcohols. In case of 10% glucose, yield of polyalcohols produced was considerably low. As for the kinds of polyalcohols formed, D-arabitol was constantly produced independently of the glucose concentration, whereas erythritol could only be produced at glucose concentrations of 30~40%, and glycerol was scarcely produced in a 10% glucose medium.

(2) Effect of nitrogen source.

Both of ammonium N and amino N are suitable for the production of polyalcohols. Now, it should be noted that in a case wherein inorganic nitrogen sources such as $(NH_4)_2SO_4$ and NH_4Cl, are employed, the pH of the medium rapidly decreases to 2.0 or lower at the initial stage of fermentation and consequently, the yield of polyalcohols formed is reduced.

Therefore, when an inorganic ammonium salt is used as the nitrogen source, a buffer solution (e.g. K-citrate-citric acid buffer[12]) must be added to prevent the depression of a pH of the medium during fermentation. (Table III)

(3) Polyalcohol production from various sugars.

As shown in Table IV, polyalcohols are remarkably produced from glucose, fructose or mannose, but scarcely formed from galactose, maltose and saccharose.

(4) Effect aeration.

When 500-ml shake-flasks containing 30 to 80 ml of

12) H. Ōnishi, This Bulletin, **21**, 143 (1957).

Table IV. Polyalcohol Production from Various Sugars by *Pichia miso*.

sugars	% of sugar consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on sugar consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
30% glucose	28.6	12.24	42.4%	###	##	###
30% fructose	26.4	8.22	31.1%	###	+	###
30% mannose	19.1	6.49	33.9%	###	+	###
30% galactose	11.3	0.12	1.1%	−	−	−
30% maltose	0.3	0.04	13.3%	−	−	−
30% saccharose	10.1	0.14	1.3%	+	−	−

Table V. Effect of Aeration on Polyalcohol by *Pichia miso*.

cultural conditions	volume of medium per flask	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
					D-arabitol	erythritol	glycerol
shaking	30 ml	27.98	12.96	46.3%	###	##	###
〃	50 ml	27.98	12.07	43.1%	###	##	###
〃	80 ml	27.65	11.91	35.3%	###	##	###
〃	120 ml	17.49	6.18	23.3%	###	±	###
〃	200 ml	11.71	2.73	23.3%	###	−	###
stationary	80 ml	5.49	1.94	35.3%	##	−	##

the medium were cultured under the above-stated conditions, polyalcohols were produced in good yields. In this case, the K_d value measured by the sodium sulfite method was 2.5×10^{-6} g mol/min. Atom. ml or higher.

However, when the flasks containing the medium exceeding 120 ml were cultured in the same conditions as mentioned above, the formation of polyalcohol was inferior to the above case. (In the case, K_d value is smaller than 1.7×10^{-6}). (Table V)

Table VI. Analtical Results of the Fermented Broth by *Pichia miso*.

% of glucose consumed	29.47%
pH	3.2
% of total polyalcohol formed (calculated as glycerol)	11.5
yield of total polyalcohol on glucose consumed (calculated as glycerol)	39.0%
% of D-arabitol formed	8.5
yield of D-arabitol on glucose consumed	28.8%
% of erythritol formed	0.6
yield of erythritol on glucose consumed	2.0%
% of glycerol formed	5.9
yield of glycerol on glucose consumed	20.0%
riboflavin formed μg/ml	2.1

[III] Isolation and purification of D-arabitol and erythritol from the fermented broth by *Pichia miso*.

(1) Analytical results of the fermented broth.

Analytical methods employed are the same as those outlined in the previous paper[1,2]. The values obtained by subtracting amounts of true glycerol from those of total polyalcohols (both calculated as glycerol), give the amount of D-arabitol and erythritol calculated as glycerol. D-Arabitol and erythritol were separately determined according to the method of Neish[6] by employing paper chromatography and subsequently eluting each of the spots with water.

The results are given in Table VI. The fermented broth showed yellow-brown colored fluorescene and the bioassay of this broth proved the formation of riboflavin, the amount of which is also given in Table VI.

(2) Isolation and purification of each polyalcohols from the fermented broth.

The methods of isolation of each polyalcohol were essentially the same as those in the previous paper[2], and the procedures are fully illustrated in Fig. 1.

From the ethanol solution, which was obtained by extracting completely dried materials of the broth, D-arabitol was obtained in crystals. However, the separation of erythritol and glycerol from each other resulted

unsuccessful though the ethanol solution was concentrated to syrup. However, successful results were obtained when the chromato-pile method that is also effectively employed in the previous paper was applied. The discs of erythritol fraction obtained in chromato-pile method were extracted with 99% ethanol by heating on a boiling water bath, and erythritol could be crystallized from this ethanol solution.

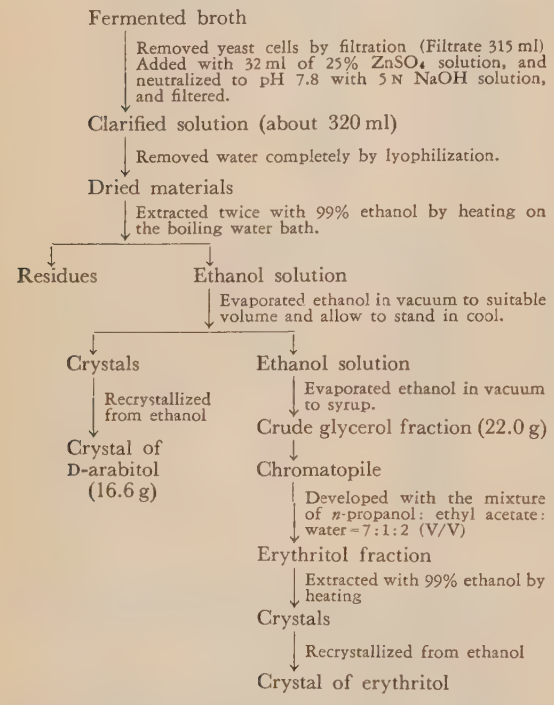

FIG. 1. Isolation and Purification of Polyalcohols from the Fermented Broth.

[IV] Identification of D-arabitol and erythritol.

Recrystallization from ethanol was carried out three times.

(1) D-Arabitol.

The D-arabitol thus obtained was prismatic crystal melting at 100.5~101.5°. This did not depress the melting point on admixture with an authentic specimen.

Anal. Found: C, 39.54%; H, 8.01%.

Calcd. for $C_5H_{12}O_5$: C, 39.41%; H, 7.89%.

$[\alpha]_D^{20} = +8.28$ (c=9.78 in saturated borax solns.)

The infrared spectra of this sample was also found to be superimposable with that of the authentic D-arabitol (Fig. 2). As illustrated in Fig. 4, the spot of this crystal agreed with that of the authentic D-arabitol.

(2) Erythritol.

The erythritol crystal obtained was tetragonal prisms melting at 119°. This did not depress the melting point on admixture with an authentic specimen.

Anal. Found: C, 39.78%; H, 7.98%.

Calcd. for $C_4H_{10}O_4$: C, 39.34%; H, 8.25%.

The infrared spectra of this sample was also found to be superimposable with that of the authentic erythritol (Fig. 3). As illustrated in Fig. 4, the spot of this crystal agreed with that of the authentic erythritol.

According to the above procedure, 13.0 g of glycerol, 1.2 g of erythritol and 19.4 g of D-arabitol were actually

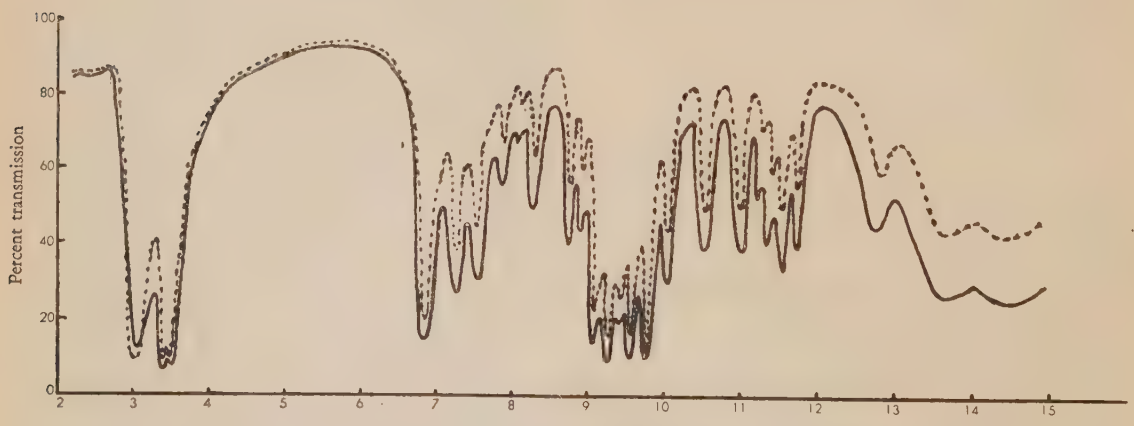

FIG. 2. Infra-red Spectra of the Crystal Obtained (D-Arabitol).

——: Crystal obtained
----: Authentic D-arabitol

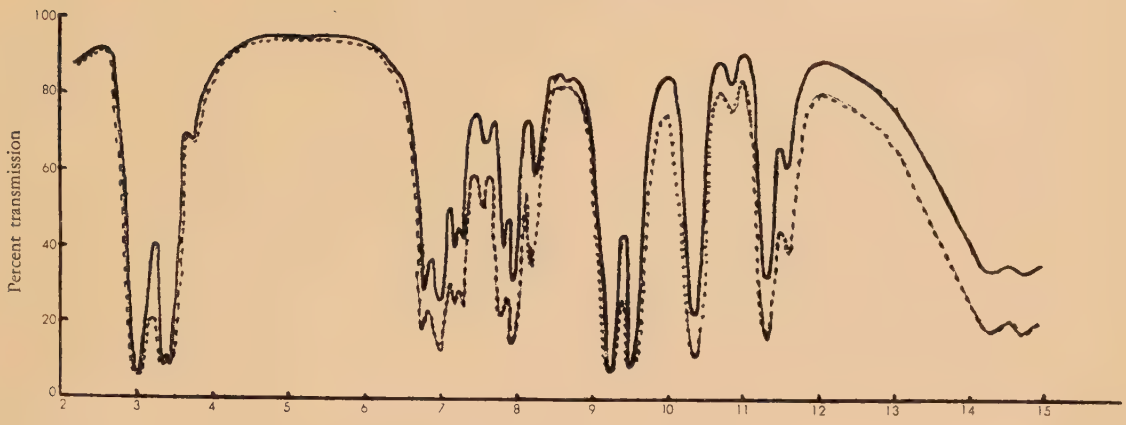

FIG. 3. Infra-red Spectra of the Crystal Obtained (Erythritol).
——: Crystal obtained
- - - -: Authentic erythritol

FIG. 4. Paperchromatogram of Crystals Obtained.
(1): Crystal obtained
(2): Authentic D-arabitol
(3): Crystal obtained
(4): Authentic erythritol

obtained from 315 ml of the fermented broth. When these values were compared with those shown in Table VI, recovery of glycerol, erythritol and D-arabitol were observed to be 69.7%, 63.4% and 72.3% respectively.

Paper chromatography of the fermentation products formed by 6 representative strains, which showed characteristic fermenting types for polyalcohol production, is illustrated in Photograph 1.

Photograph 1. Characteristic Fermenting Types for Polyalcohol Production by 6 Representative Strains.

(1) *Saccharomyces sake*, producing glycerol alone.
(2) *Trigonopsis variabilis*, producing erythritol alone.
(3) *Debaryomyces sake*, producing D-arabitol alone.
(4) *Pichia miso*, producing glycerol, erythritol and D-arabitol
(5) *Saccharomyces rouxii*, producing glycerol and D-arabitol.
(6) *Torulopsis famata*, producing erythritol and D-arabitol.

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2, p. 140~146, 1960]

Studies on L-Glutamic Acid Fermentation

Part II. Fermentative Production of L-Glutamic Acid from Glucose by *Brevibacterium divaricatum* nov. sp.

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Basic researches performed with the purpose of finding a better industrial method to produce L-glutamic acid has resulted in the successful isolation of *Brevibacterium divaricatum* nov. sp.

The conditions of fermentation production of L-glutamic acid employing *Brevibacterium divaricatum* have been examined.

After a series of experiments with moderate culture constituents under proper conditions for fermentation, it became possible to obtain 45 mg/ml broth of L-glutamic acid after 30 hours' incubation taking glucose as the substrate, feeding a proper amount of urea aqueous solution during the process in order to keep the culture medium weak alkaline, offering nitrogen at the same time.

The yield of L-glutamic acid was exceeded 45% on the base of initial glucose supplied.

All experimental details are discussed in this paper.

INTRODUCTION

In recent years, special interest has been directed to the production of L-glutamic acid by a fermentation method in view of its importance as a flavoring agent, which has a large commercial demand.

In a previous paper[1], both morphological and physiological properties of a new group of strain No. 1627, which as the only producing L-glutamic acid was isolated in a good quantity, have been examined. The name, *Brevibacterium divaricatum* nov. sp. was proposed to this strain

1) Y.C. Su and K. Yamada, This Bulletin, **24**, 69 (1960).

Table I. Influence of Nitrogen Sources on the Production of L-Glutamic Acid.

No.	N-Source	N-Compound		N-Compound concentration	Yield of L-G. A.	
		M. W.	N%		mg/ml broth	%
1	NH_4Cl	53.50	26.17	1.42	7.4	14.8
2	NH_4NO_3	80.05	35.00	1.06	6.1	12.2
3	$(NH_4)_2SO_4$	132.14	21.20	1.76	12.2	24.4
4	KNO_3	101.10	13.86	2.69	0.7	1.4
5	$NaNO_3$	85.01	16.47	2.26	0.9	1.8
6	$NaNO_2$	69.01	20.29	1.82	—	—
7	$NH_4H_2PO_4$	115.04	12.17	3.06	13.0	26.0
8	NH_4-tart.	184.15	15.25	2.45	18.6	37.2
9	Urea	60.06	46.70	0.80	17.3	34.6

N-source corresponding to approximately 0.37% N.

because it was believed to be fallen under the head of a new species of genus *Brevibacterium* after it was contrasted with Bergey's Manual, 7th Ed.

The present paper deals with the accumulation of L-glutamic acid from glucose by *Brevibacterium divaricatum* nov. sp.

A detailed study of culture conditions favoring the accumulation of L-glutamic acid will be reported in this paper, and a discussion concerning the mechanism of L-glutamic acid formation is also presented.

EXPERIMENTAL AND RESULTS

Microorganisms—*Brevibacterium divaricatum* was used through-out the experiments.

Analytical Methods—For the quantitative analysis of L-glutamic acid, the manometric method using glutamic decarboxylase preparation obtained from *E. coli* najjar was adopted[2].

Glucose was determined by Somogyi-Shaffer-Hartman's method[3].

The extent of cell growth was known after measuring its optical density using a Beckman electric spectrophotometer (562 mμ).

To estimate α-ketoglutaric acid and pyruvic acid the colorimetric method of Friedemann-Haugen was applied[4].

Succinic acid was determined by the manometric method using succinic acid dehydrogenase obtained from pig heart muscle[5]; while, for the determination of alanine, a bioassay method using *Leuconostoc citrovorum*[6] was employed.

The Influence of the Nitrogen Source and its Concentration on the Production of L-Glutamic Acid.

Nine different nitrogen sources, as listed in Table I, including $(NH_4)_2SO_4$ and urea, were employed to test the effect of the nitrogen source.

For this experiment, the basal medium was composed of 5.0 g glucose, 0.1 g K_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.2 g peptone and 0.2 g meat extract in 100 ml of distilled water (initial pH, 7.0).

Culture were grown in 80 ml of the medium, in 500-ml flasks and incubated at 30°C on a reciprocating shaker at a rate of 112 o. p.m. (oscillations per minute). After 72 hours' incubation, the culture fluids were filtered off and the filtrates were subjected to analysis.

The results obtained are as follows (Table I):

It was found that NH_4-tartarate and urea are excellent nitrogen sources for L-glutamic acid accumulation.

Among the inorganic nitrogen sources, ammonium salts proved to be a good nitrogen source for L-glutamic acid fermentation of this strain; particularly, the non-assimilability of nitrate and nitrite was found to be interesting.

The concentration of the nitrogen sources was more important for acid production than their nature. For instance, when $(NH_4)_2SO_4$ and urea were used, the optimal concentration was observed to be 1.5~2.0% and 0.67~0.9%, respectively, (corresponding to 0.31~0.42% N), and rather sharp peak seemed to appear at these levels. A remarkable decrease in acid production resulted at concentrations either higher or lower than

2) W. W. Umbreit and I. C. Gunsalus, *J. Biol. Chem.*, **159**, 333 (1945).
3) P. A. Schaffer and M. Somogyi, *J. Biol. Chem.*, **100**, 695 (1933).
4) T. E. Friedemann and G. E. Haugen *J. Biol. Chem.*, **147**, 415 (1943).
5) W. W. Umbreit, R. H. Burris and S. F. Stauffer, Manometric Techniques and Tissue Metabolism, 166~169 (1951).
6) H. E. Sauberlich, *J. Biol. Chem.*, **177**, 545 (1949).

TABLE II. INFLUENCE OF GLUCOSE CONCENTRATION.

No.	Glucose concentn. (mg/ml)*	After 72 hours' incubation		
		Residual glucose (mg/ml)	L-G. A. (mg/ml)	The corresponding highest yield (%)
1	50	—	15.8	31.6
2	75	—	22.8	30.4
3	100	—	26.5	26.5
4	125	2.1	28.2	22.5
5	150	20.0	25.7	17.1

* Amount of urea added
No. 1……0.67 % No. 2……1.0 % No. 3……1.3 % No. 4……1.6 % No. 5……1.9 %

TABLE III. EFFECT OF GROWTH SUBSTANCE ON THE PRODUCTION OF L-GLUTAMIC ACID.

Growth substance	Substance added	L-Glutamic acid (mg/100 ml)	Comparative ratio
Control	—	2430	1.00
Casate	0.2 %	2580	1.06
Peptone	0.2	2630	1.08
Corn steep liquor	0.2	2730	1.12
Meat extract	0.2	2850	1.17
Yeast extract	0.2	2820	1.16
Bran extract	3.0	2970	1.22
Meat extract Bran extract	0.2 3.0	3060	1.26

Incubation: 72 hours, in shaken culture at 30°C.

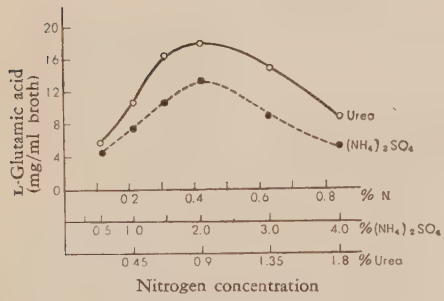

FIG. 1. Influence of Nitrogen Concentration on Production of L-Glutamic Acid.

the above levels (Fig. 1).

The Influence of Carbohydrate Concentration on the Production of L-Glutamic Acid.

Glucose was employed as the substrate; the basal medium was composed of 0.1 g K_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.2 g peptone, 0.2 g meat extract and urea (the proportion between supplied glucose and amount of urea added are shown in Table II) in 100 ml of water.

Incubation was carried out for 72 hours at 30°C. The results are as follows (Table II):

It was observed that the optimal initial concentration of glucose seemed to lie in the range of 5.0~10.0%, and beyond this range the higher the concentration, the lower the yield. However, the acid yield might be affected by the feeding of nitrogenous substances, so conclusions can not be drawn from this experiment alone.

The Effects of Various Growth Substances on L-Glutamic Acid Production.

In this experiment the basal medium was composed of 10% glucose, 1.3% urea, 0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$. To this medium various growth substances shown in Table III were added.

After 72 hours' incubation, quantitative determinations of L-glutamic acid were carried out (Table III).

This experiment demonstrated that bran extract, meat extract, yeast extract and corn steep liquor give a considerable stimulative effect to L-glutamic acid accumulation, whereas casate and peptone do not have any noticeable effect.

In one case the addition of meat and bran extracts resulted in increment of acid by 26%. A possible explanation for this case is that in those extracts there exist some stimulative essences suitable for enzymic ac-

TABLE IV. EFFECT OF METALLIC IONS ON THE PRODUCTION OF L-GLUTAMIC ACID.

No.	Metallic ions	Compound added		pH	L-G. A. (mg/100 ml)	Comparative ratio
1	Control	——*		5.7	1460	1.00
2	Na^+	NaCl 1×6^{-1} M		5.2	1310	0.89
3	K^+	KCl 1×6^{-1} M		5.8	1440	0.98
4	Mg^{++}	$MgCl\cdot6H_2O$	0.02 μM	5.4	1620	1.11
5	Mn^{++}	$MnSO_4\cdot7H_2O$	//	5.4	1260	0.86
6	Fe^{++}	$FeSO_4\cdot7H_2O$	//	5.8	1820	1.24
7	Fe^{+++}	$Fe_2(SO_4)_3$	//	5.5	1834	1.25
8	Co^{++}	$CoSO_4\cdot7H_2O$	//	5.4	1420	0.97
9	Ni^{++}	$NiSO_4\cdot7H_2O$	//	5.4	1530	1.05
10	Cu^{++}	$CuSO_4\cdot5H_2O$	//	5.4	1397	0.95
11	Zn^{++}	$ZnSO_4\cdot7H_2O$	//	5.3	1300	0.89
12	Mo^{6+}	Na_2MoO_4	//	5.2	1716	1.17
13	Cd^{++}	$CdSO_4\cdot7H_2O$	//	5.2	875	0.59

* Med.: glucose 5%, urea 0.8%, K_2HPO_4 0.1%, $MgSO_4\cdot7H_2O$ 0.05%, meat extract 0.2%, bran extract 3.0%. Incubation: 72 hours, in shaken culture at 30°C.

tion.

The Effects of Metals on the Production of L-Glutamic Acid.

It has been reported by Asai et al.[7] that in *Serratia marcesens*, the addition to the medium of 0.02 μM/ml of iron as ferrous sulfate and ferric sulfate resulted in a marked stimulation of α-ketoglutaric acid accumulation. This suggests that Fe^{++} and Fe^{+++} most probably maintain an equilibrium state between themselves during fermentation and promote the accumulation of acid.

The authors therefore examined the effect of various metals including iron on L-glutamic acid accumulation by the addition to cultures of *Brevibacterum divaricatum*.

The effects of metals are summarized in Table IV.

Molybdenum and iron gave a marked stimulation, but the effects of cadmium and manganese were somewhat low, and the other metals tested had no noticeable influence.

Effect of Incubation Temperature on the Growth of Cells and L-Glutamic Acid Production.

In this experiment the basal medium composition was 10 g glucose, 1.3 g urea, 0.1g K_2HPO_4, 0.05 g $MgSO_4\cdot7H_2O$, 0.2 g meat extract and 3 ml bran extract in 100 ml of distilled water. Eightly ml of sterilized medium in 500-ml flasks were inoculated with a suspension of cells from a bouillon agar slant and incubated at 25°C, 30°C and 37°C on a reciprocating shaker at a rate of 112 o.p.m.

After 24, 48 and 72 hours, the broth sample was taken out to determine both the quantity of produced L-glutamic acid and the extent of cell growth. The results are shown in Fig. 2.

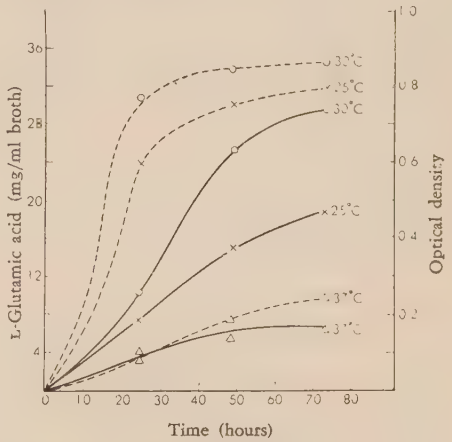

FIG. 2. Effect of Incubation Temperature on the Growth of Cells and L-Glutamic Acid Production.

—— L-glutamic acid
--- growth of cells

As clearly shown in Fig. 2, the time required for cell growth to arrive at a stationary phase was about 24~30 hours in shaken cultivation kept at 30°C. The L-glutamic acid accumulated accordingly increased reaching a maximum after 72 hours.

Both cell growth and amount of L-glutamic acid produced were far from satisfactory when incubation temperature was held at 37°C.

When the incubation temperature was maintained at

7) T. Asai, K. Aida, Z. Sugisaki and N. Yakeishi, *J. Gen. Appl. Microbiol.*, **1**, 317 (1955).

25°C, the L-glutamic acid yield was not so high as in case the temperature was maintained at 30°C, though growth of cells seemed to be normal.

Effect of K_d on the Production of L-Glutamic Acid.

In order to investigate the influence of oxygen on L-glutamic acid formation the following experiments were performed.

Maintaining the temperature at 30°C, a medium having the same constituents as that employed in the previous experiment was used for cultivation under the five conditions given below:

No. 1: Surface culture was performed with 100 ml of medium in a 500-ml Erlenmeyer flask.

No. 2: Using a reciprocating shaker (131 o. p. m.), 100 ml of medium in a 500-ml shaking flask was shaken cultivated. ($K_d=7.31\times10^{-6}$g.mole of O_2/atm.min.ml)

No. 3: Using a reciprocating shaker (112 o. p. m.), 100 ml of medium in 500-ml shaking flask was shaken cultivated. ($K_d=5.13\times10^{-6}$)

No. 4: 80 ml of medium in 500-ml shaking flask was cultivated by a reciprocating shaker (112 o. p. m.). ($K_d=5.5\times10^{-6}$).

No. 5: 100 ml of medium in a 500-ml shaking flask was cultivated by a reciprocating shaker (95 o. p. m.) ($K_d=0.94\times10^{-6}$)

Yield of L-glutamic acid after 72 hours' incubation is shown in Fig. 3.

It was observed that the aerobic condition is preferable for cell growth and acid production. The recommended oxygen supply was $5\times10^{-6}\,K_d$.

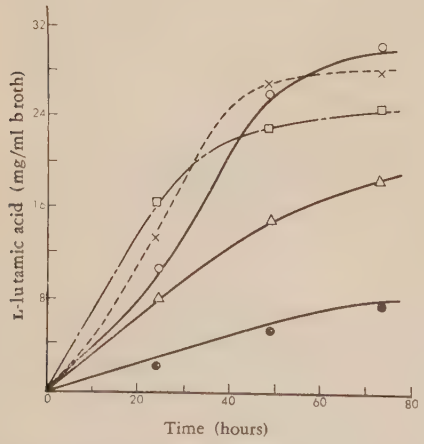

FIG. 3. Effect of K_d on the Production of L-Glutamic Acid.

○——○ $K_d=5.13\times10^{-6}$ ×---× $K_d=5.50\times10^{-6}$
□——□ $K_d=7.13\times10^{-6}$ △——△ $K_d=0.94\times10^{-6}$
●——● surface culture

On L-Glutamic Acid Fermentation by the Feeding Method.

By adding the nitrogen source continuously or semi-continuously during fermentation while transformation of the substrate to the product is taking place, the total concentration of the nitrogen source in the fermenter is enhanced to a certain extent, resulting in a high efficiency of production provided that the microorganisms are physiologically tolerant to the resulting environmental conditions.

For this purpose, a 20 l jar fermenter was used. To control the culture condition sampling was performed at a fixed time. The urea aqueous solution was feeded now and then not only in order to supply the nitrogen but also to maintain the medium slightly alkaline. Influences on L-glutamic acid production were then examined.

The main culture was composed of 10% glucose, 0.3% urea, 0.1% K_2HPO_4, 0.05% $MgSO_4\cdot7H_2O$, 0.2% meat extract, 3% bran extract and 0.15% corn steep liquor (initial pH 7.0).

Incubation was performed in a 20 l jar fermenter containing 14 l of medium. Sterile air was introduced at a rate of 1/4 vol. per volume of the medium per minute. The stirring rate was 600 r. p. m. and the incubation temperature was maintained at 30°±1°C.

Four portions of 100 ml of 45% urea aqueous solution were feeded one after another to the culture medium at 9, 11.5, 14 and 17 hours after incubation. After 21.5, 24, 27 and 30 hours, four portions of 50 ml of 45% urea aqueous solution were again feeded. The results are shown in Fig. 4.

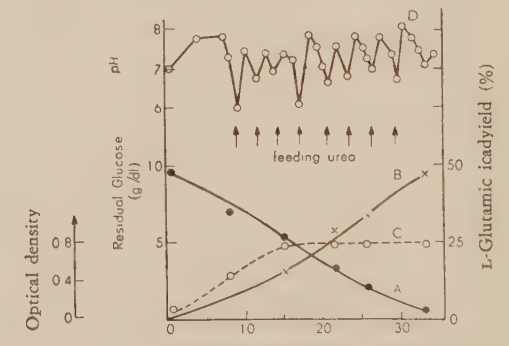

FIG. 4. The Effect of Feeding Urea on L-Glutamic Acid Production.

A: residual glucose
B: L-glutamic acid yield
C: growth of cells
D: pH

As shown in Fig. 4, it was found that periodical urea feeding to supply a proper quantity of nitrogen source at a fixed time not only largely accelerated the consumption of glucose but also increased the accumulation of L-glutamic acid to a noticeable degree.

Periodical Determination of L-Glutamic Acid during Fermentation.

As a conclusion of the series of experimental results, developments of fermentation are presented in Table V and Fig. 5.

The experimental procedure was as follows. A culture medium identical with that of the previous experiment, was employed to observe the effect of urea feeding on L-glutamic acid production. A culture of 5 l of medium containing 10% glucose with basal components was grown in a 10 l glass jar fermenter attached with a stirrer. Sterile air was introduced at a rate of 0.25 vol. per volume of the medium per minute. The stirring rate was 1000 r.p.m. and incubation temperature was maintained at 30°C.

Beginning at about 25 hours after incubation was started cell growth proceeded to a stationary phase, and the production of L-glutamic acid began to increase following the formation of α-ketoglutaric acid.

TABLE V. RESULTS OF L-GLUTAMIC ACID PRODUCTION BY AN EXPERIMENTAL JAR FERMENTER.

Hours	pH	Optical density	Residual glucose (g/dl)	L-G. A. (g/dl)	α-K-G* (g/dl)	Pyruvic acid (g/dl)	Succinic acid (g/dl)	Alanine (g/dl)
0	6.9	0.026	10.49	—		—	—	—
12	7.4	0.57	7.47	0.55	0.050	0.065	0.154	0.035
18	7.5	0.75	4.97	1.64	0.077	0.093	0.300	0.091
25	8.0	0.93	1.32	3.18	0.106	0.053	0.292	0.202
27	7.2	0.95	0.55	3.68	0.119	0.021	0.187	0.201
29	8.2	0.94	0.12	4.40	0.103	0.008	0.093	0.261
30	8.3	0.95	0.11	4.42	0.097	0.006	0.077	0.249

* α-K-G indicates α-ketoglutaric acid.

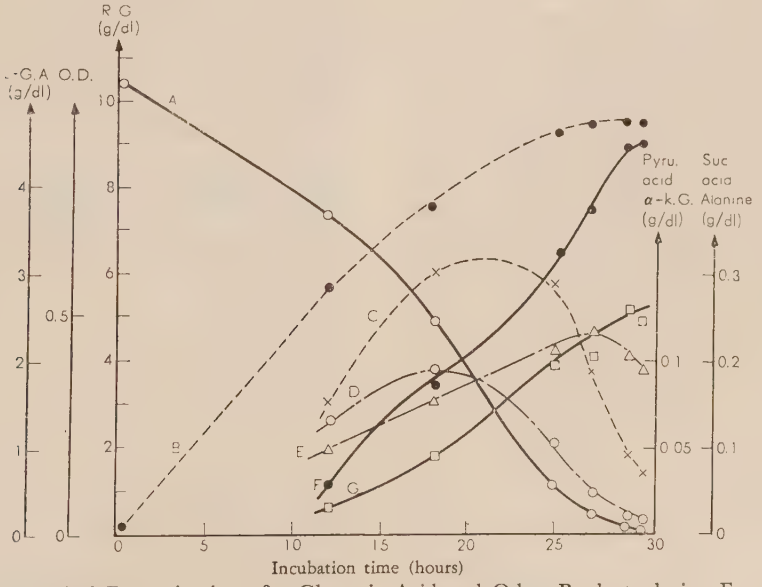

FIG. 5. Periodical Determination of L-Glutamic Acid and Other Products during Fermentation.

A: Residual glucose
B: Growth of cells (Optical density)
C: Succinic acid
D: Pyruvic acid
E: α-Ketoglutaric acid
F: L-Glutamic acid
G: Alanine

After 29 hours' incubation the acid formed was 45% on the basis of glucose supplied.

The phenomenon that the yield of alanine is roughly proportional to the amount of pyruvic acid consumed is considered to be due to the inversion of most of the pyruvic acid to alanine when ammonia is accompanied.

By observing the quantity and kinds of fermentation products and the time when they are formed, L-glutamic acid was presumed to be originated from α-ketoglutaric acid which was formed from pyruvic acid via Krebs TCA cycle. Whereas, pyruvic acid was assumed to come from glucose via the Embden-Meyerhof-Parnas scheme or another pathway.

SUMMARY

(1) With regard to the constituents of the fermentation medium, various nitrogen sources, glucose and their optimum concentrations, as well as the additive influences of various growth substances and metals, were examined.

Solely considering its worth in industrial application, urea seems to be a satisfactory nitrogen source. Its optimum concentration in the medium containing 5% of glucose was found to lie between 0.67 to 0.9%. The growth of cells should be suppressed when the initial concentration of glucose exceeds 12.5%; therefore a level of 10% gives a better result. Data showed that addition of meat and bran extracts gave an increment of about 26% in acid yield.

(2) As for the fermentation condition, after experiments on the incubation temperature, pH and the influence of oxygen supply, etc., reveal that the aerobic condition is favorable for cell growth and accumulation of L-glutamic acid. An oxygen supply around 5×10^{-6} of K_d is desirable. The optimum incubation temperature was found to lie at 30°C.

A reasonable quantity of L-glutamic acid will be produced if the culture is kept neutral or preferably slightly alkaline by feeding a proper amount of urea aqueous solution throughout incubation.

(3) As a conclusion of the above experimental results, the yield of L-glutamic acid reached 45% on basis of glucose supplied after 30 hours' incubation.

(4) By-products such as alanine, α-ketoglutaric acid, pyruvic acid and succinic acid were found at the same time in the fermentation of L-glutamic acid employing *Brevibactcrium divaricatum* nov. sp.

By observing the time of formation, the kind and the amount of these by-products it was presumed that the L-glutamic acid formation with this strain most probably proceeds from glucose to pyruvic acid, and then to α-ketoglutaric acid via Krebs TCA cycle. L-Glutamic acid could be considered to be formed from α-ketoglutaric acid. The reductive amination reaction of α-ketoglutaric acid is assumed to be the main pathway of L-glutamic acid synthesis.

Acknowledgement The authors wish to express their sincere thanks to Prof. T. Asai for his kind advice throughout this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2, p. 147~152, 1960]

A New Basic Triple Salt Containing Magnesium Hydroxide

Part III. The Quinary System KCl-K_2SO_4-$MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O at 100°

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The quinary system KCl-K_2SO_4-$MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O and associated systems KCl-K_2SO_4-$Mg(OH)_2$-H_2O and $MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O were investigated at 100°. Upon the accomplishment of this quinary system, it was proved that the crystallization field of the new basic triple salt (NS salt B) was so large that various bitterns produced in salt manufacturing factories are included in this field, and it thus became possible to directly separate potassium as sulfate from bittern at 100°.

INTRODUCTION

The 100° isotherm of systems KCl–K_2SO_4–$Mg(OH)_2$–H_2O, $MgCl_2$–$MgSO_4$–$Mg(OH)_2$–H_2O and KCl-K_2SO_4-$MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O have been studied. The equilibrium of this quinary system at 100° has been accomplished in the present and the previous two reports[1,2]. Besides this work disputations were also made on the only one report[3] concerning the 100° isotherm of quaternary system KCl–K_2SO_4–$MgCl_2$-$MgSO_4$–H_2O, excluding magnesium hydroxide from this quinary system.

The crystallization field of NS salt B($K_2SO_4 \cdot 2MgSO_4 \cdot Mg(OH)_2 \cdot 2H_2O$) in this quinary system was so large that it was able to easily separate this salt from various solutions included in this system and moreover, as the solubility of this salt was comparatively small, the yield of this salt was also high.

To date, potassium dissolved in bittern has been separated as carnallite or potassium chloride because no direct method of separation of potassium as sulfate has ever been established. At present however this has become possible as a result of the discovery of NS salt B and the accomplishment of the isotherm of this quinary system.

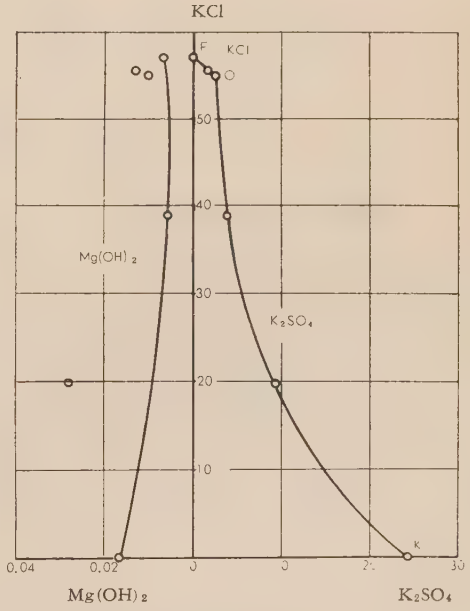

FIG. 1. 100° Isotherm of the System KCl-K_2SO_4-$Mg(OH)_2$-H_2O. (g/100 g H_2O)

1) J. Sugi and M. Nakayama, This Bulletin, **22**, 208 (1958).
2) M. Nakayama, *ibid.*, **23**, 46 (1959).
3) A.N. Campbell, K.W. Downes and C.S. Samis, *J. A. C. S.*, **56**, 2507 (1934).

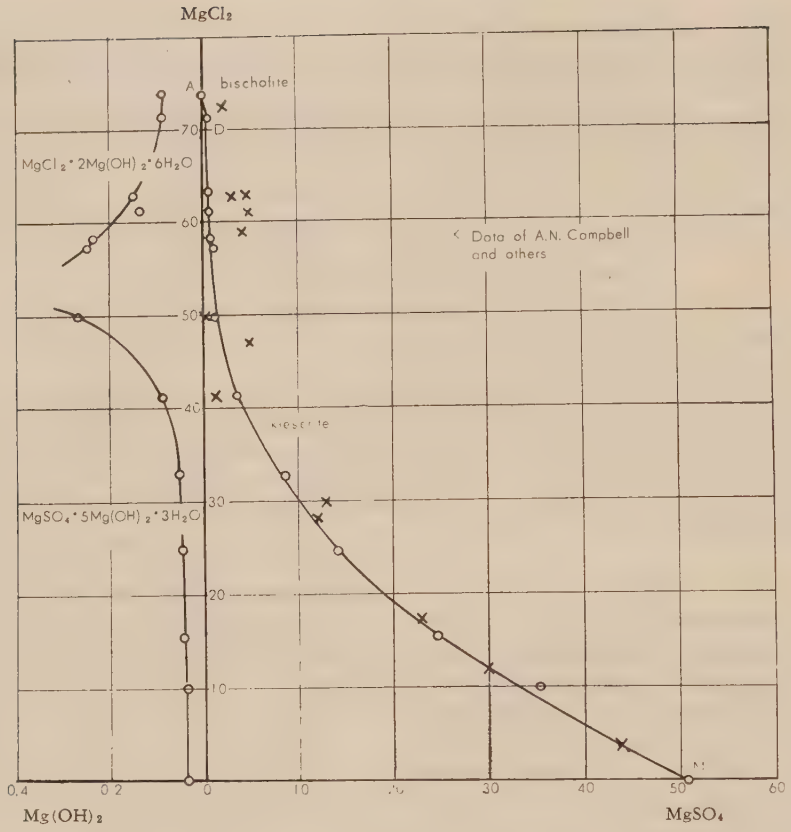

FIG. 2. 100° Isotherm of the System $MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O. (g/100 g H_2O)

RESULTS AND DISCUSSION

Experimental details were the same as described in the previous papers[1,2].

System KCl–K_2SO_4–$Mg(OH)_2$–H_2O at 100° Heretofore though nothing has been reported about this system, in concern of the 100° isotherm of ternary system KCl–K_2SO_4–H_2O, excluding magnesium hydroxide from the system, some papers such as those of H. Precht and B. Wittjen[4], W. C. Blasdale[5], E. Cornec and H. Hering[6] and A. N. Campbell and others[3] have been published.

Data obtained from this system are shown in Fig. 1. The solubility of magnesium hydroxide in this system was so small that the diagram must be divided into two, namely those of potassium chloride-potassium sulfate and those of potassium chloride-magnesium hydroxide.

The chemical compositions of the liquid phase of monovariant point O, whose solid phases were potassium chloride, potassium sulfate and magnesium hydroxide, were as follows:

KCl, 54.69; K_2SO_4, 2.531; $Mg(OH)_2$, 0.0102 g/100 g H_2O

These compositions agreed with those obtained by H. Precht and B. Wittjen[4], W. C. Blasdale[5] and E. Cornec and H. Hering[6], but did not coincide with those results obtained by A. N. Campbell and others[3].

4) H. Precht and B. Wittjen, *Ber.*, **14**, 1674 (1881).
5) W.C. Blasdale, *J. Ind. Eng. Chem.*, **10**. 344 (1918).
6) E. Cornec and H. Hering, *Caliche*. **8**, 58 (1926).

System $MgCl_2$–$MgSO_4$–$Mg(OH)_2$–H_2O at 100° Concerning this quaternary system, the equilibrium diagram of any temperature has not yet been reported. Concerning the 100° isotherm of ternary system $MgCl_2$–$MgSO_4$–H_2O, excluding magnesium hydroxide from this quaternary system, only one report has been published by A. N. Campbell and others[3]. According to their report, in the region which the concentration of magnesium chloride was high, anhydrous magnesium sulfate was deposited by the dehydration of magnesium chloride. This conclusion was however doubtful, because no report referring that anhydrous magnesium sulfate is observed at 100° from any system like this ternary system except their report has ever appeared and moreover, the determinations of magnesium sulfate in the region of a high concentration of magnesium chloride were largely dispersed (cf. Fig. 2) and the chemical compositions of monovariant point, co-existent point of two kinds of salts anhydrous magnesium sulfate and kieserite, are not shown.

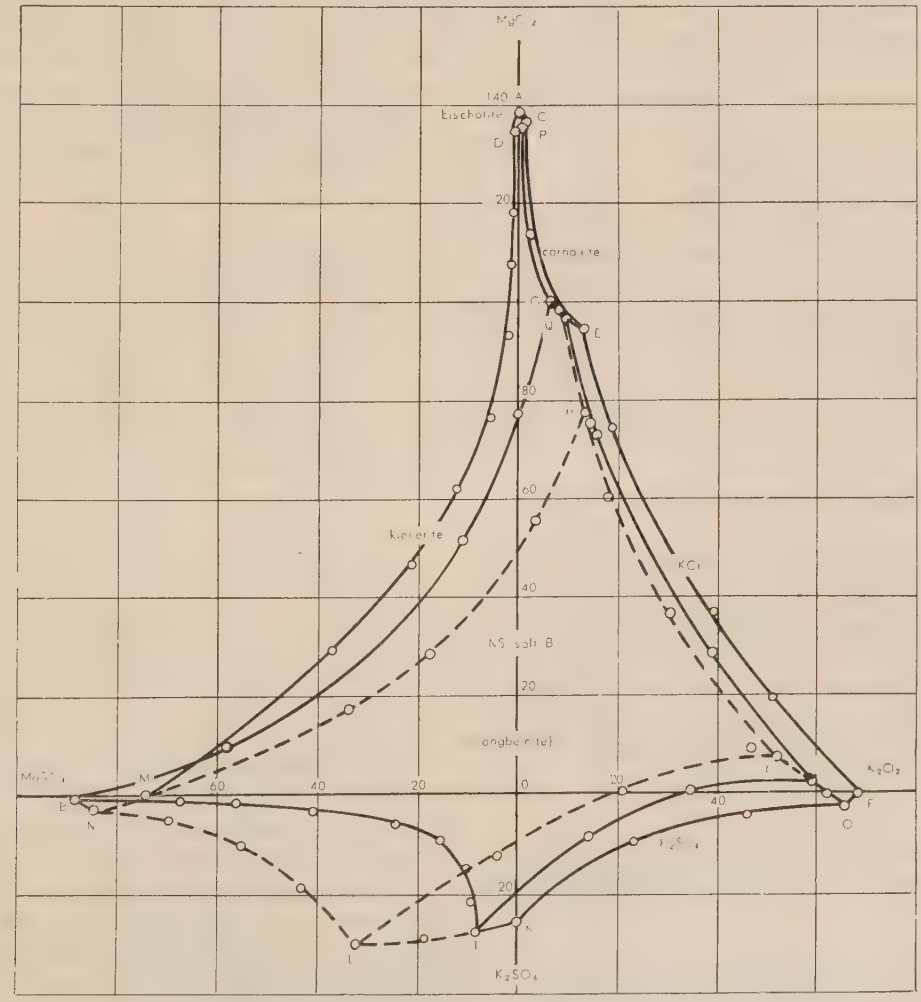

FIG. 3. 100° Isotherm of the System K_2Cl_2-K_2SO_4-$MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O. (mol/1000 mol H_2O)

Therefore, the examination was carried out with caution of the following facts: (1) whether anhydrous magnesium sulfate is crystallized or not, (2) solubility of magnesium sulfate in the region of high concentration of magnesium chloride, (3) solubility of magnesium hydroxide, etc.

Data obtained from this system are presented in Fig. 2. The solubility of magnesium hydroxide in this system was relatively large as compared with those of other systems in the series of this study, but it was not so large as to permit the three components to be drawn in one figure; therefore the diagram must be divided into two parts, namely those of magnesium chloride-magnesium sulfate and those of magnesium chloride-magnesium hydroxide.

As shown in the left hand of Fig. 2, the solubility of magnesium hydroxide in this system, taking the concentration of magnesium chloride as the parameter, closely resembled the solubiliy curve of magnesium hydroxide in the foregoing system, $MgCl_2$–$Mg(OH)_2$–H_2O. It is therefore reasonable to consider that the solubility of magnesium hydroxide in this system is chiefly influenced by the concentration of magnesium chloride.

The solubility of magnesium sulfate in such a region as the concentration of magnesium chloride is higher than 50 gram per 100 gram of water becomes very small, but anhydrous magnesium sulfate formation does not occur and the monohydrate of magnesium sulfate is the stable solid phase of all regions in the presence of the depositing anhydrous magnesium sulfate reported by A. N. Campbell and others[3]. This fact is also confirmed by the powder X-ray diffraction method.

The chemical compositions of the monovariant point D, co-existent point of three kinds of salts, bischofite, kieserite and $MgCl_2 \cdot 2Mg(OH)_2 \cdot 6H_2O$, were

$MgCl_2$, 71.37; $MgSO_4$, 0.417; $Mg(OH)_2$, 0.0846 g/100 g H_2O

System KCl–K_2SO_4–$MgCl_2$–$MgSO_4$–$Mg(OH)_2$–H_2O at 100° Any report concerning this quinary system have never been published. On the 100° isotherm of quaternary system KCl–K_2SO_4–$MgCl_2$–$MgSO_4$–H_2O, excluding magnesium hydroxide from this quinary system, a report was made by A. N. Campbell and others[3], but they seemed to have made several serious errors as previously been pointed out in this study. Therefore, the isotherm of quaternary system as well as the quinary system were studied.

Results obtained from both quinary and quaternary systems are shown in Table I and represented graphically in Figs. 3 and 4. The broken lines in these figures represent the equilibrium of the quaternary system. As the amount of dissolved magnesium hydroxide was too small to be indicated, plotting was omitted in these figures.

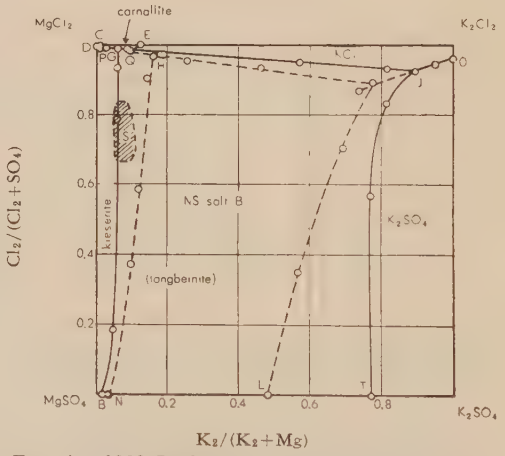

FIG. 4. 100° Isotherm of the System K_2Cl_2-K_2SO_4-$MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O. (mol)

The crystallization field of NS salt B being very large as shown in Figs. 3 and 4, it should be possible to easily separate this salt from the solutions included in this quinary system and moreover, comparing the yield of NS salt B with those of langbeinite, the former should be greater than the latter because the saturation surface of the former is located below those of the latter.

The range of chemical compositions of various

TABLE I. DATA FOR 100° ISOTHERM OF THE SYSTEM
KCl-K_2SO_4-$MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O

Points of Fig. 3 and 4	Composition of Liquid Phase (mol per 1000 mol H_2O)				Agitation period hrs.	Solid Phase Remarks**
	Mg	Cl_2	SO_4	$(OH)_2$		
F	0.021	68.26	—	0.0207	168	Kc+$Mg(OH)_2$
E	96.77	108.11	—	1.822	96	Kc+car.+B. M. C.
C	137.40	138.46	—	0.2678	264	bis.+car.+B. M. C.
A	139.37	139.13	—	0.2622	460	bis.+B. M. C.
D	135.91	135.02	0.624	0.2613	336	bis.+kie.+B. M. C.
M	76.16	—	76.05	0.1072	72	kie.+B. M. S.
K	0.052	—	25.07	0.0516	96	Ks+$Mg(OH)_2$
O	0.032	66.08	2.617	0.0315	240	Kc+Ks+$Mg(OH)_2$
T	7.947	—	35.55	0.0074	—	Ks+NS.B+B. M. S.
	9.600	24.20	18.26	*1	72	Ks+NS.B
	10.26	45.25	9.252	*2	576	〃
J	7.779	66.75	5.534	*3	96	Kc+Ks+NS.B
	3.582	65.81	3.416	*4	336	Kc+Ks+$Mg(OH)_2$
	32.35	71.45	3.764	0.0340	168	Kc+NS.B
Q	99.00	107.90	1.203	0.9216	144	Kc+car.+NS.B
	101.21	108.60	0.740	0.6779	360	car.+NS.B
G	101.77	106.60	0.646	0.9057	408	car.+NS.B+kie.
	115.06	117.65	0.352	0.3893	360	car.+kie.+B. M. C.
P	137.22	138.84	0.351	—	408	bis.+kie.+car.+B. M. C.
	82.92	82.48	5.466	0.1388	96	kie.+NS.B+B. M. S.
	67.16	56.21	15.57	0.0164	96	〃
	71.62	13.46	61.70	0.0191	98	〃
B	87.33	—	88.05	0.2041	312	〃
L	31.82	—	61.82	—	—	Ks+NS.B+lang.
	22.94	18.55	34.33	—	192	〃
	16.10	37.62	15.53	*5	120	〃
I	16.61	65.73	7.652	—	336	Kc+Ks+lang.+NS.B
	13.25	65.54	4.977	*6	120	Ks+NS.B+lang. (supersatd.)
	19.46	66.90	9.674	*7	148	Kc+NS.B+lang.+Ks (〃)
	42.37	73.22	5.445	0.0031	192	Kc+NS.B+lang.
	64.82	82.93	4.087	0.0011	216	〃
	78.58	93.11	2.779	0.0498	140	〃
H	80.95	94.57	2.954	—	144	Kc+kie.+lang.+NS.B
	62.86	64.93	6.770	—	192	kie.+lang.+NS.B
	54.35	36.07	25.71	—	192	〃
	57.04	23.55	39.58	—	192	〃

*1~*7 pH values were as follows:
*1 pH=7.50/22°C
*2 pH=7.05/22°C
*3 pH=7.12/21°C
*4 pH=7.22/22°C
*5 pH=7.20/21.5°C
*6 pH=6.91/21.5°C
*7 pH=7.10/21°C

** Chemical formulae and names of solid phases are abbreviated as follows:
Kc=KCl, Ks=K_2SO_4, kie.=kieserite $MgSO_4 \cdot H_2O$, bis.=bischofite $MgCl_2 \cdot 6H_2O$, car.=carnallite $KCl \cdot MgCl_2 \cdot 6H_2O$, lang.=langbeinite $K_2SO_4 \cdot 2MgSO_4$, NS.B=NS salt B $K_2SO_4 \cdot 2MgSO_4 \cdot Mg(OH)_2 \cdot 2H_2O$, B.M.C.=Basic Magnesium Chloride $MgCl_2 \cdot 2Mg(OH)_2 \cdot 6H_2O$, B. M. S.=Basic Magnesium Sulfate $MgSO_4 \cdot 5Mg(OH)_2 \cdot 3H_2O$

bittern produced by salt manufacturing factories are shown in Fig. 4 by "S". The range "S" is included in the crystallization field of NS salt B, but not in those of langbeinite, therefore, NS salt B could be directly separated from bittern, while langbeinite could not be separated directly.

From these experimental data, in order to separate potassium dissolved in bittern as sulfate directly at 100°, it should be concluded that the only one method is to separate it as NS salt B. Calculating the yield of NS salt B from bittern at 100° by these phase diagrams, about fifty per cent of dissolved potassium is separated as NS salt B.

From the equilibrium diagram of the quaternary system, eliminated magnesium hydroxide from the quinary system, our experimental data hardly agreed with results obtained by A. N. Campbell and others[3].

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2, p. 152~159, 1960]

Studies on Taka-α-amylase (8)*

On the Mechanism of EDTA** Inactivation of TAA***

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This paper deals with some properties of reversibly inact.**** TAA effected by means of EDTA. The mechanism of EDTA inactivation was discussed from the view point that EDTA has a conspicious suppressive effect on the reactivation of heat-inact. enzyme, while it has no effect on the active enzyme itself. Particular attention was paid to the problem of calcium transfer along with the progress of inactivation.

INTRODUCTION

The role of metal-binding reagents such as EDTA, on the inactivation of a variety of α-amylases has been ascribed to the removal of some calcium ions which are essentially incorporated into enzyme proteins. Stein and Fischer[1] have however reported that α-amylases of different origins including *Aspergillus oryzae* are quite stable towards the EDTA action if the enzyme preparations are completely free from the contaminated proteases.

The reversible inactivation of α-amylases by means of EDTA has been reported earlier by Fischer and Haselbach employing malt-α-amylase[2].

* Presented at the 154- and 156th Meeting of the Agri. Chem. Soc. of Japan held in Kyoto, Nov. 15, 1958 and Jan. 17, 1959.

** Ethylenediamine tetracetate-Na.

*** Taka-α-amylase

**** inactivated

1) E. A. Stein and E. H. Fischer, *J. Biol. Chem.*, **232**, 867 (1958).

2) E. H. Fischer and C. H. Haselbach, *Helv. Chim. Acta*, **34**, 325 (1951).

In the previous report, the reactivation phenomena of EDTA-inact. TAA was studied, and the equilibrium between the active and inact. TAA was described[3].

Recently, Yamamoto and Hukumoto have succeeded in obtaining the EDTA-inact. pancreas-, or bacterial-α-amylase in a completely reversible state, and have emphasized the importance of metal ions for restoring activity[4].

This report deals with one assumptive schema regarding the role of EDTA which plays a part during inactivation process.

$$\underset{(\mathrm{Ca}\ 1\ \mathrm{mol})}{\text{active TAA}} \overset{\text{heat}}{\rightleftarrows} \underset{(\mathrm{Ca}\ 1\ \mathrm{mol})}{\text{inact. TAA}} \overset{\text{EDTA}}{\rightleftarrows} \underset{(\mathrm{Ca}\ 1\ \mathrm{mol})}{\text{inact. TAA}} \longrightarrow \underset{(\mathrm{Ca}\ ?)}{\text{irreversibly denatured}}$$

Yamamoto et al. have also pointed out on the special behavior of TAA towards EDTA and suggested a similar schema for the EDTA inactivation of TAA[5].

METHODS AND MATERIALS

Crystalline TAA was prepared from Taka-diastase Sankyo according to Akabori's method[6]. After recrystallization for 3 times, innert calcium was removed by the author's method[7]; Rivanol-amylase complex was crystallized by shaking a concentrated solution of the complex in M/2 acetate buffer (pH 6.0) at 20°C[8]. Rivanol was removed by caolin, and crystallization was executed in M/5 acetate buffer (pH 6.0) by means of acetone. The crystals thus obtained contained about 1 mol of calcium[7].

The enzymes were always diluted to M/25,000 before use. The determination of enzyme concentrations was based on the assumption that the molecular weight of TAA should be 53,000[9].

The relative change of amylase activity was followed by the percentage of saccharifying power remaining during the course of incubation with EDTA. Saccharifying power was assayed by a slightly modified method of Willstätter and Schudel[10]; one ml of M/25,000 TAA was diluted to 200 ml, and this solution was used as a standard for comparison.

Assayed samples were previously controlled to have almost equal activity to the standard (error within $\pm 5\%$) and then the iodine consumption was compared as a basis for calculation. Sampling and measurement of activity were always carried out at pH 5.0 in order to prevent the reactivation of activity[3]. The assay system employed was: 2% soluble starch solution 20 ml containing M/50 acetate buffer of pH 5.0, enzyme solution 5 ml, 35°C, ten minutes.

RESULTS

1. The Reversibility of the Inactivation.

Fig. 1 illustrates the reversible inactivation of TAA induced by incubation with EDTA. One ml of each M/5,000 EDTA solution of various pH (M/20 veronal buffer) was added to a series of tubes containing 1 ml of the enzyme solution, and then incubated at 32.5°C*. The reactivation was effected by adding 2 ml of M/100 calcium acetate solution and M/10 veronal buffer (pH 8.5), respectively, and incubating them at the same temperature from one to two days. The dotted lines show the course of restoring activity.

As a further example of showing the reversibility of the enzyme, inactivation was performed in almost the same way as that given in Fig. 1 (37°C, pH 8.5, 100 mol equivalent of EDTA), and reactivation was merely effected by disposure to a lower temperature (Fig. 2).

After restoration of the activity reached a maximum level, the mixture was again warmed up to 37°C. The enzyme lost its activity again almost in accordance with the first course of inactivation (dotted line).

Salts of bivalent metals, such as calcium acetate, magnesium sulfate and barium chloride were of considerable potency in regenerating the enzyme and, to a lesser extent, manganese, cobalt, and zinc were also effective. The degree of potency ran parallel with the metal complex strength of EDTA (i.e., Ca>Mg>Ba), but, as

3) A. Tanaka, *J. of Japanese Biochem. Soc.*, **30**, 431 (1958).
4) T. Yamamoto and J. Hukumoto, This Bulletin, short communication, **23**, 68 (1959).
5) T. Yamamoto and J. Hukumoto, Presented at the 155th Meeting of the Agri. Chem. Soc. Japan, held in Osaka, Dec. 13, (1958).
6) S. Akabori, T. Ikenaka and B. Hagihara. *J. Biochem.*, **41**, 577 (1954).
7) A. Tanaka. *Science Report of the Hyogo University of Agriculture*, **2**, 84 (1956).
8) A. Tanaka. *J. of Japanese Biochem. Soc.*, **28**, 529 (1956).
9) S. Akabori et al., *J. of Biochem.*, **41**, 803 (1954).
10) R. Willstätter and G. Schudel, *Ber.*, **57**, 780 (1918).
* In all of the following experiments, controls without added EDTA were quite stable.

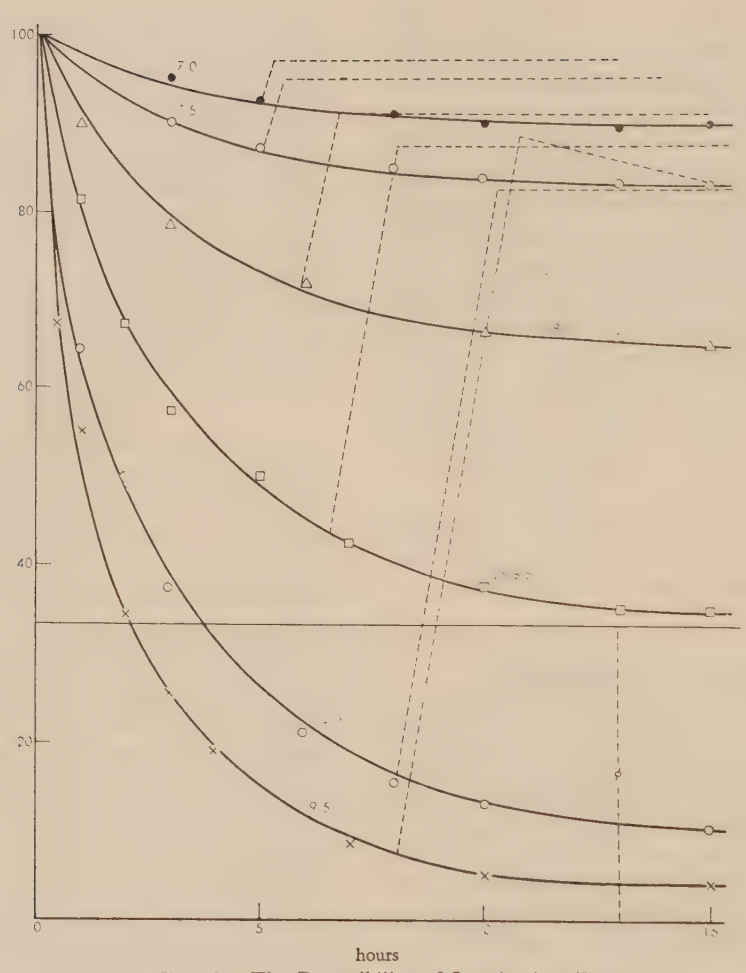

FIG. 1. The Reversibility of Inactivation (I)

Dotted lines do not show the time course of restoring the activity, but they only show the maximum levels that can be reached by incubation with calcium acetate solutions.

for heavy metals, this relation was disturbed by the denaturing action of heavy metals themselves.

2. Effect of the Dilution upon the Rate of Restoring Activity.

Partially inactivated enzyme which was prepared in almost the same way as described in Exp. 1 was diluted to a various extent with M/40 veronal buffer (pH 8.5), and then kept in a refrigerator to effect reactivation. The progress of restoring activity was measured by the passage of time (Tables Ia and Ib). In the latter case, calcium acetate solution was added previously prior to dilution in order to elliminate the influence of excess EDTA. In both cases, the rate of reactivation was not affected practically by the degree of dilution, suggesting that the reactivation is of a type of monomolecular reaction.

3. Effect of EDTA on the Reactivation of EDTA-inact. TAA and Heat-inact. TAA.

One ml aliquot of M/250 EDTA solution (pH

8.5, M/20 veronal buffer) was added to a series of tubes containing 1 ml of the enzyme stock solution, and then the mixture was incubated at 37°C. After three hours, the reaction mixtures were withdrawn, were kept at 6°C to effect reactivation, 1 ml of EDTA or calcium solution being added beforehand to control their concentrations in the solution (Table II).

In cases where heat-inact. enzymes were employed, EDTA solutions were added to the heat-

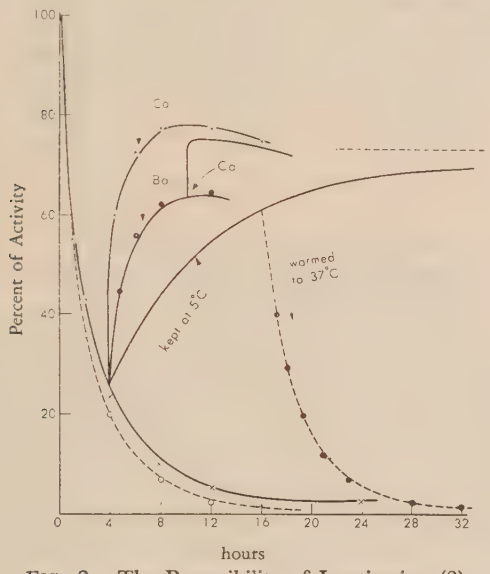

FIG. 2. The Reversibility of Inactivation (2)

Barium- and Calcium-salt solutions (1 ml, M/100, pH 8.5) were added merely for comparison. Dotted lines at the right hand side was displaced leftwards at the intact form .

TABLE I. EFFECT OF THE DILUTION UPON THE RATE OF RESTORING ACTIVITY

a. diluted in the presence of unreacted EDTA

dilution	incubation periods at 6°C (hrs.)			
	0	12	24	48
1	21.2	52.5	61.5	70.4
5	21.2	52.5	61.5	70.4
10	21.2	51.2	62.5	70.0

Inactivation condition: 37°C, pH 8.5 (veronal buffer M/40) 4 hrs, EDTA 100 mol equivalent

b. diluted after cancelling the unreacted EDTA with calcium acetate.*

dilution	incubation periods at 6°C.			
	0	5	24	48
1	47.7	72.0	79.5	85.0
10	47.7	73.4	81.5	85.9
50	47.7	73.5	80.0	85.9

Inactivation condition: 37°C, pH 8.5, 2 hrs, EDTA 100 mol equiv.

* Calcium acetate solution was added so as to make the final concentration of Ca^{++} to M/1,000.

TABLE II. INFLUENCE OF THE ADDITION OF EDTA AND CALCIUM UPON THE REACTIVATION OF EDTA-INACT. TAA

additions	incubation period at 6°C (days)				
	0	3	5	7	check*
M/100 $Ca(Ac)_2$	32.3	90.0	86.5	83.5	86.9
H_2O**	32.3	63.0	57.2	55.9	82.6
M/50 EDTA	32.3	41.5	27.7	20.4	76.5
M/10 EDTA	32.3	32.5	21.5	15.5	76.0

* After seven days 1 ml of 1M $Ca(Ac)_2$ solution was added and was stood at 6°C for 3 days.

** The net concentration of EDTA is calculated to be about M/1,000.

TABLE III. THE SUPPRESSIVE ACTION OF EDTA TOWARDS THE REACTIVATION OF HEAT-INACT. TAA

a. incubated at 5°C

EDTA added*	incubation periods (hrs.)			
	0	25	50	check**
0.0	34.0	63.5	68.0	69.1
2.5	34.0	59.0	67.5	69.2
25.0	34.0	48.0	50.7	69.2
250.0	34.0	40.0	42.8	69.9

b. incubated at 20°C

EDTA added*	incubation periods (hrs.)			
	0	2	4	check**
0.0	33.0	56.0	63.0	66.0
2.5	33.0	55.0	61.8	67.7
25.0	33.0	36.5	36.8	67.0
250.0	33.0	33.5	31.8	67.1

c. incubated at 28°C

EDTA added*	incubation periods (hrs.)			
	0	1	2	check**
0.0	32.5	52.3	60.0	64.1
1.0	32.5	45.0	59.2	65.9
2.5	32.5	41.5	51.5	66.0
5.0	32.5	35.5	39.0	66.0

* Expressed by mol equivalent of TAA.

** Occurrence of irreversible inactivation was checked up. 2 ml of M/100 $Ca(Ac)_2$ solution was added and was incubated at 28°C for 20 hrs.

TABLE IV. pH DEPENDENCE OF THE SUPPRESSIVE ACTION OF EDTA TOWARDS THE REACTIVATION OF HEAT-INACTIVATED ENZYME.

pH	activities after 25 hrs. incubation at 5°C*		difference	check**	
	EDTA added (A)	not added (B)	($B-A$)	(A')	(B')
6.0	49.1	52.5	3.4	58.7	57.3
8.0	45.5	55.5	10.0	62.9	60.0
8.5	43.0	58.3	15.3	64.7	62.0
9.0	38.0	56.5	18.5	62.9	62.8
9.5	37.9	57.3	19.4	64.1	65.5

* Activity at zero hour was 29.4.
** 2 ml aliquots of M/100 $Ca(Ac)_2$ solution were added to A and B, and held at 6°C for 2 days.

inact. enzymes (inactivated by 50°C, 1 hr., pH 8.5, M/20 veronal buffer), and incubation was carried out at different temperature stages (Table IIIa~c).

It is of special interest to note the temperature dependency of the suppressive ability of EDTA. As for heat- inact. enzyme, for example, as much as 250 M EDTA was required to stop the reactivation at 5°C, and 25 M at 20°C, but only 5 M EDTA was enough to stop their self-reactivation at 28°C.

Table IV shows the pH dependency of the suppressing ability of EDTA. One ml aliquot of the enzyme stock solution was adjusted to pH 8.5 by 1 ml of M/20 veronal buffer, and incubated at 50°C for 1.5 hrs. This procedure destroyed about 70% of the activity. After cooling, 1 ml of M/100 EDTA solution was added, pH being adjusted beforehand by 5 ml of M/10 veronal buffer, and incubated at 5°C for twenty-five hours. Controls without added EDTA were simultaneously run.

The difference may give the measure of the suppressing power of EDTA at that pH. Enhanced suppression can be observed as the pH value rises.

Throughout these experiments, no loss of activity due to the reversible and irreversible denaturation was observed. For example, incubation of active amylase with 100 mol equivalent of EDTA at pH 8.5 deprived only 5.0 and 6.5% of their activities upon incubation at 5°C for seven days and at 20°C for four hours, respectively. As for irreversible inactivation, note the checks given in the Tables.

4. Calcium Content of the Enzyme reactivated by means of Magnesium Ion.

TAA solution (8×10^{-5} M, 8.5 ml, about 360 mg dry matter) was incubated at 33°C for seven hours with 10 mol equivalent of EDTA (M/50 solution 3.4 ml), pH of the solution being adjusted by the addition of 10 ml of veronal buffer of pH 9.5. By this procedure about 80% of activity was destroyed. Seventeen ml of M/50 $MgSO_4$ solution was then added to this solution, and it was kept at 28°C overnight, about 88% of the original activity being recovered thereby.

Enzymes were collected by precipitation with acetone, and were then converted into a crystalline Rivanol-amylase complex[8] (166.6 mg dry matter). The complex was then subjected to assay of calcium and magnecium. Results of flame-photometrical analysis of mineral components of the specimen after being dried over P_2O_5 at 56°C in vacuum till a constant weight was obtained, was as follows:

Found: Calcium 82.4 γ (which corresponds to about 0.75 mol equiv. per 1 mol equiv. of amylase protein).
Magnesium trace (below 10 γ)

5. Effect of EDTA on the Rate of Irreversible Inactivation.

One ml aliquot of the enzyme stock solution was mixed with each 1 ml of M/10 veronal buffer (pH 8.5) and M/1,000 EDTA solution (25 M equiv. of EDTA), held at 38°C for 13.5 hours, the enzyme almost completely losing its activity thereby. One ml of each ascending concentra-

tion of EDTA solutions was then added anew to the inact. enzyme solution, and incubation was continued again at the same temperature. The reaction mixtures were then withdrawn at intervals of several hours, and to these was added 1 ml of M/100 calcium acetate solution, and incubated at 28°C overnight to effect reactivation.

The results obtained are shown in Fig. 3.

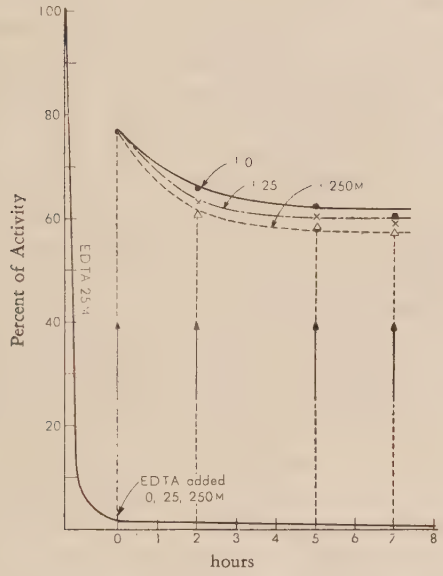

FIG. 3. Effect of the Concentration of EDTA on the Rate of Irreversibilization

Under curve: TAA activities were diminished by incubation with 25 M equiv. of EDTA. After being almost completely destroyed, 0, 25, and 250 M equiv. of EDTA were added, and the incubation was continued.

Upper curve: Enzymes were reactivated by incubation with an excess calcium acetate solution at 28°C. Three curves show the corresponding decrease in the amount of the remaining reversibly inact. TAA during EDTA digestion.

The results show that the concentration of EDTA is of a secondary significance in regard of the rate of irreversiblization, at least in so far as the concentration of EDTA does not overwhelmingly predominate. Looking at the matter having these facts in mind it may be suspected that the irreversibilization much more depends on a reaction of a self-decomposing nature.

DISCUSSION

In the last report, a kinetic scheme for a partially reversible system has been presented as the mechanism of EDTA inactivation of TAA[3].

Let k_1, k_2 and k_3 be the velocity constants of the forward, reverse and irreversibly denatured reactions, respectively, then:

$$\underset{\text{active}}{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{\text{inactive}}{B} \xrightarrow{k_3} \underset{\text{inactive}}{C} \qquad (1)$$

An example of a further confirmation of schema (1) may be found in the following data showing the formation of inflection points. To follow the net change in activity after complete restoration, i.e., $(A+B)$ with time, the partially inactivated enzyme which was withdrawn at time-intervals shown in Fig. 4, was incubated with a slight excess of the calcium acetate solution, and activity recovered was measured. The corresponding graphical analysis is shown in Fig. 4. Apparent inflection points can be seen in curves A′, B′, and C′.

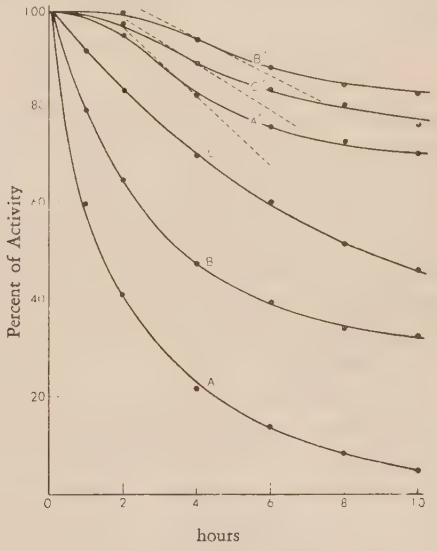

FIG. 4. Formation of the Inflection Point

Curves A, B, C and A′, B′, C′ show the course of EDTA-inactivation before or after restoring their activities by incubation with Ca-acetate solution. Inactivation conditions are

A) 100 M equiv. of EDTA, 37°C, pH 8.5 (M/40 Veronal buffer)
B) EDTA 2.5M, 37.5°C, pH 8.0.
C) EDTA 5.0 M, 35°C, pH 8.0.

A mathematical analysis of this phenomenon will be demonstrated as follows:

From the schema (1) we get

$$A=\frac{A_0}{K_2-K_1}\{(K_2-k_1)e^{-K_1t}+(k_1-K_1)e^{-K_2t}\}$$

$$B=\frac{k_1A_0}{K_2-K_1}(e^{-K_1t}-e^{-K_2t})$$

then $A+B=\frac{A_0}{K_2-K_1}(K_2e^{-K_1t}-K_1e^{-K_2t})$

where, A_0: initial amount of active amylase

$$K_1=\frac{\sum_{i=1}^{3}k_i-\sqrt{(\sum_{i=1}^{3}k_i)^2-4k_1k_3}}{2}$$

$$K_2=\frac{\sum_{i=1}^{3}k_i+\sqrt{(\sum_{i=1}^{3}k_i)^2+4k_1k_3}}{2}$$

In order to follow the time-change of $(A+B)$, put

$$f(t)=K_2e^{-K_1t}-K_1e^{-K_2t}$$

and differentiating it successively with respect to t gives:

$$f'(t)=K_1K_2(e^{-K_2t}-e^{-K_1t})<0$$
$$f''(t)=K_1K_2(K_1e^{-K_1t}-K_2e^{-K_2t})$$

Let $f''(t)$ be zero, then we get the inflection point at

$$t_{\text{infl.}}=\frac{1}{K_2-K_1}\log\frac{K_2}{K_1}\quad(\because\ K_2>K_1)$$

Focussing our attention upon the convexity of the curve—

let $t<t_{\text{infl.}}$ then $f''(t)<0$ (upper convex)

let $t>t_{\text{infl.}}$ then $f''(t)>0$ (under convex)

The shape of the curve expected from these results is quite in agreement with the tendency of the curve shown in Fig. 4, and it can thus also be taken as evidence of showing reliability of the mechanism.

As our next step in analysis, let us enter into some details concerning the mechanism of EDTA inactivation.

If we consider the most probable process which can be expected from schema (1), then

a) Amylase·Ca (active)
+EDTA $\rightleftarrows$ amylase (inact.)
+Ca·EDTA $\longrightarrow$ irreversibly inactivated

b) Amylase·Ca (active)
+EDTA $\rightleftarrows$ amylase·Ca·EDTA (inact.)
$\longrightarrow$ irreversibly inactivated

Assuming that the mechanism of inactivation is either of the two types pictured above, the loss of activity would then have to follow the course of a bimolecular reaction.

As it has been already shown the enzyme lost its activity, quite unexpectedly being due to monomolecular reaction, for example, raising the concentration of EDTA over a certain limit did not accompany a corresponding increase in the rate of inactivation[11]. Such mechanisms must therefore be excluded, aud further studies are required to obtain a better schema compatible with the experimental data.

It is now a well-known fact that some parts of the enzyme in the solution are present in reversibly-inactivated state (probably inactivated by heat), and are in an equilibrium state with the remaining active enzyme.

In cases where the temperature is low enough so as to stabilize the enzyme, the equilibrium is thought to be shifted overwhelmingly towards the active side, and much of the largest part of the enzyme in the solution exists in the active form.

Here, in this case the results of Exp. 3 have showed that the reactivation of heat-inact. enzyme is predominantly inhibited by the presence of EDTA. This indicates that the addition of EDTA to this equilibriated system (active $\rightleftarrows$ heat inact.) may result in stopping the backward (reactivating) reaction, followed by an equivalent loss in enzyme activity. Such a speculation may lead us to one plausible hypothesis that it is only the heat-inact. enzyme in solutions which is influenced by the sequestering action of EDTA.

Supporting evidence can be obtained by comparing the pH dependences of deactivation with the ability of EDTA to stop the regeneration (Table IV).

A rough proportionality can then be observed between them, that is, in the pH range so far as EDTA can act effectively, the degree of deactivation is highly correlated with the ability to stop the regeneration of heat-inact. enzyme.

11) A. Tanaka, *J. of Japanese Biochem. Soc.*, **28**. 567 (1956).

However, the question still remains open as to whether some addition compounds of heat-inact. enzyme with EDTA may occur or whether or not the Ca atom may be removed from the heat-inact. enzyme by EDTA.

Experiments designed to explore these points have shown that the reactivated enzyme, which was effected without the addition of calcium salts, still contains at least 1 mol of calcium (Fig. 2 dotted line and Exp. 4), and neither calcium nor some other essential factor is required in the process of regeneration (Exp. 2). In other words, this type of regeneration is of a self-reactivating nature, and 1 mol of calcium still remains to be removed, at least at the stage of reversible inactivation.

The results of Exp. 4 may give satisfactory evidence to illustrate the role of bivalent metal ions for restoring activity: that is, the addition of bivalent metal ions including magnesium ion have no direct interaction with the protein moiety of amylase, but only play a part in cancelling the EDTA from their surroundings, thus facilitating the self-reactivation of the enzyme.

It would therefore conclusively be postulated that the mechanism of EDTA inactivation might mainly consist in the accumulation of heat-inact. enzyme through the sequestering action of EDTA. On this basis, a formulation of the following mechanism is suggested:

$$\underset{\text{active}}{A} \overset{\text{heat}}{\rightleftarrows} \underset{\text{inactive}}{A'} \overset{\text{EDTA}}{\rightleftarrows} \underset{\text{inactive}}{B} \longrightarrow \underset{\text{inactive}}{C}$$

The complex formation of the heat-inact. enzyme with EDTA would indeed be expected to be the cause of accumulation of the heat inact. enzyme, but no clear evidence was obtained in this work.

Acknowledgement. The author is indebted to Professor S. Yoshimura for his help in carrying out this experiment, to Mr. T. Yamamoto for his stimulating discussions, and to Miss. F. Stanley for her kind revision.

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Studies on Phenolic Lactones

Part III. Synthesis of (±) Hibalactone

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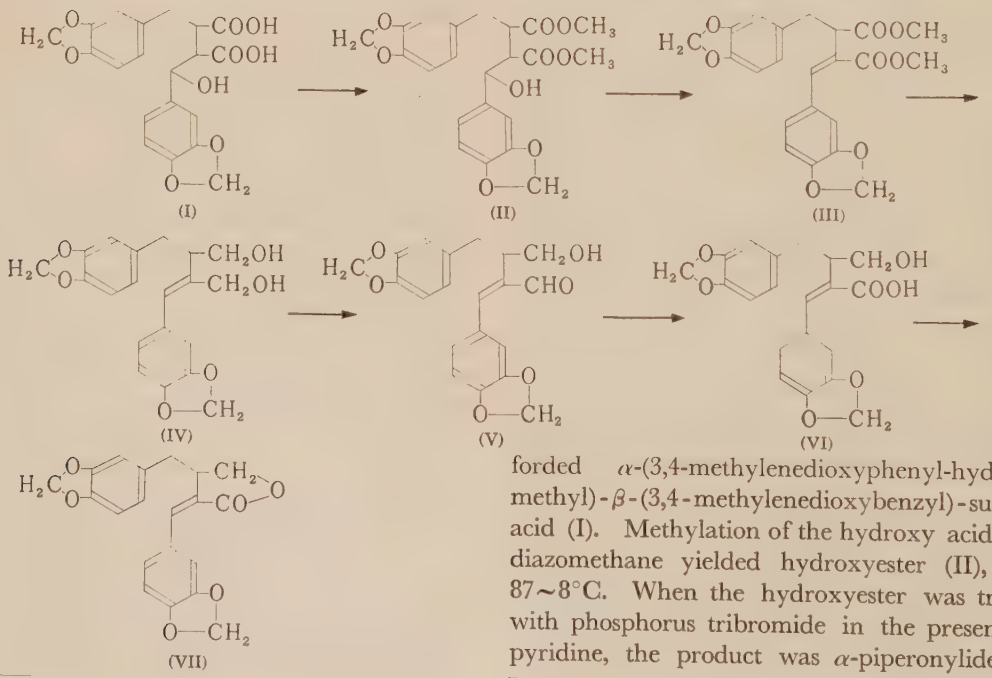
(±) Hibalactone, α-piperonylidene-β-piperonyl-butyrolactone, was synthesized by the following method. α-Piperonylidene-β-piperonyl-succinic acid dimethylester was reduced with lithium aluminum hydride to 2-piperonylidene-3-piperonyl-1, 4-butanediol, m. p. 117°C. Oxidation of the glycol with manganese dioxide in acetone yielded α-piperonylidene-β-piperonyl-γ-hydroxybutyraldehyde, which afforded racemic hibalactone, m.p. 154~5°, after oxidation with silver oxide and lactonization.

In the previous paper the authors reported the synthesis of isohibalactone, the geometric isomer of hibalactone, by two methods; (1) reduction of α-piperonylidene-β-piperonyl-succinic acid β-thioethyl α-methyl ester with Raney nickel catalyst[1], (2) condensation of piperonal with β-piperonyl-butyrolactone[2]. Authors devised the other method for synthesizing α-piperonylidene-β-piperonyl-butyrolactone, and synthesized racemic hibalactone as follows:

The condensation of piperonal and dimethyl piperonyl-succinate with sodium ethylate afforded α-(3,4-methylenedioxyphenyl-hydroxymethyl)-β-(3,4-methylenedioxybenzyl)-succinic acid (I). Methylation of the hydroxy acid with diazomethane yielded hydroxyester (II), m.p. 87~8°C. When the hydroxyester was treated with phosphorus tribromide in the presence of pyridine, the product was α-piperonylidene-β-



1) K. Yamashita and M. Matsui, This Bulletin, **22**, 277 (1958).

2) K. Yamashita and M. Matsui, This Bulletin, **23**, 230 (1959).

piperonyl-succinic acid dimethylester (III), m.p. 83~4°C, and not bromoester. Reduction of dimethyl ester (III) with lithium aluminum hydride afforded 2-piperonylidene-3-piperonyl-1, 4-butanediol (IV), m.p. 117°C. When this glycol was treated with precipitated manganese dioxide powder in acetone the hydroxymethyl group adjuscent to double bond was oxidized to aldehyde group and α-piperonylidene-β-piperonyl-γ-hydroxy-butyraldehyde (V) was obtained. Oxidation of the aldehyde with silver oxide in alkaline solution afforded hibalactonic acid (VI), m.p. 143~4°C, and lactonization with hydrochloric acid afforded racemic hibalactone (VII), m.p. 154~5°C. The lactone showed good agreement in infrared spectrum and ultraviolet spectrum with authentic (−)hibalactone.

The authors express their thanks to Dr. Mitsuo Masumura of Tokushima University, for kindly supplying the authentic (−)hibalactone.

EXPERIMENTAL

All melting points were observed value only.

α-(3,4-Methylenedioxyphenyl-hydroxymethyl)-β-(3, 4-methylenedioxybenzyl)-succinic acid dimethylester (II).

α-(3,4-Methylenedioxyphenyl-hydroxymethyl)-β-(3,4-methylenedioxybenzyl)-succinic acid[1], 13 g, was suspended in 50 ml of ether and esterified with ethereal solution of diazomethane prepared from 10 g of nitrosomethylurea. Evaporation of solvent left 13.5 g of ester, m.p. 85~7°C. After recrystallization from methanol, its melting points rose to 87~8°C. *Anal.* Found: C, 60.74; H, 5.08. Calcd. for $C_{22}H_{22}O_9$: C, 61.4; H, 5.1%.

α-Piperonylidene-β-piperonyl-succinic acid dimethyl ester (III).

α-(3,4-Methylenedioxyphenyl-hydroxymethyl)-β-(3, 4-methylenedioxybenzyl)-succinic acid dimethyl ester, 13 g, was dissolved in 30 ml of benzene and 0.12 g of pyridine was added. To a solution of 2.95 g of phosphorus tribromide in 50 ml of benzene, 0.5 g of pyridine was added and the above ester solution was added dropwise with stirring in the cold. After refluxing 2 hours, the reaction mixture was poured onto ice and extracted with ether. After washing twice with water, evaporation of the solvent from ethereal extract left 12 g of α-piperonylidene-β-piperonyl-succinic acid dimethyl ester, m.p. 83~4°C. Recrystallization from ethyl acetate-petroleum benzine afforded colorless needles, m.p. 84~5°C. *Anal.* Found: C, 63.96; H, 4.66. Calcd. for $C_{22}H_{20}O_8$: C, 64.1; H, 4.8%.

2-Piperonylidene-3-piperonyl-1, 4-butanediol (IV).

A solution of 11 g of dimethyl α-piperonylidene-β-piperonyl-succinate in 200 ml of dry ether was added dropwise to a suspension of 1.3 g of lithium aluminum hydride in 50 ml of anhydrous ether at a rate so as to produce gentle reflux. After refluxing for an hour, the excess hydride was destroyed by careful addition of ethyl acetate and water. With stirring 40 ml of 15% hydrochloric acid was slowly added. The ether layer was separated and the solvent was removed by distillation under atmospheric pressure. The residual oil was refluxed with 50 ml of 5% methanolic potash for an hour to destroy any unreacted ester. After the addition of 50 ml of water, methanol was distilled off and glycol was extracted with ether. Evaporation of ether left 8.9 g of oily glycol, which gradually solidified, m.p. 108~9°C. Recrystallization from ethanol gave colorless needles, m.p. 115~7°C. *Anal.* Found: C, 66.51; H, 5.18. Calcd. for $C_{20}H_{20}O_6$: C, 67.4; H, 5.6%.

(±)-Hibalactone.

To a solution of the glycol, 8.9 g, in 150 ml of acetone precipitated manganese dioxide powder (35 g) was added and left standing for 24 hours with occasional shaking. Manganese dioxide was filtered and washed with acetone, and the combined filtrate was concentrated to syrup. α-Piperonylidene-β-piperonyl-γ-hydroxy-butyraldehyde was obtained as a oily residue, weighed 8.0 g. Chloroform could be used in place of acetone but acetone was more preferable as the solvent.

The crude aldehyde (8.0 g) was dissolved in 50 ml of warm ethanol and 80 ml of 5% sodium hydroxide was added. Silver oxide freshly precipitated from 8.0 g of silver nitrate was added and warmed for an hour

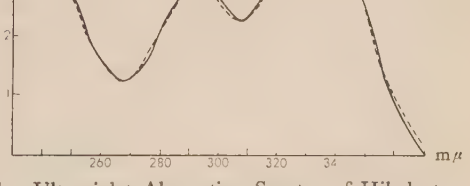


FIG. 1. Ultraviolet Absorption Spectra of Hibalactone.
—— Synthetic (±) Hibalactone
---- Authentic (−) Hibalactone

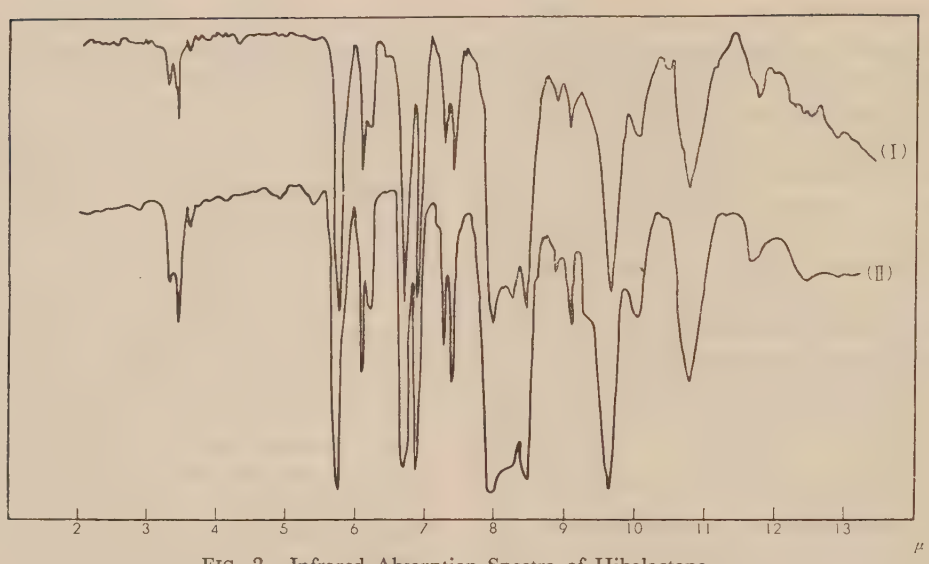


FIG. 2. Infrared Absorption Spectra of Hibalactone.
(I) Authentic (−) Hibalactone
(II) Synthetic (±) Hibalactone

with vigorous stirring. The hot mixture was filtered and after the addition of 100 ml of water ethanol was distilled off. Upon cooling the sparingly soluble sodium salt of hibalactonic acid was precipitated gradually. After the removal of neutral substances with chloroform extraction, the aqueous solution was acidified with hydrochloric acid and warmed on water bath for 30 minutes. The reaction mixture was carefully poured into excess of sodium bicarbonate solution and extracted three times with chloroform. The combined extract was washed with water and dried over sodium sulfate. After evaporation of chloroform the residual mass was dissolved in 100 ml of hot ethanol and cooled in refrigerator. The precipitated crude hibalactone was collected by filtration and recrystallized from methanol, yielded 3.1 g, m.p. 154°C. *Anal.* Found: C, 67.52; H, 4.81. Calcd. for $C_{20}H_{16}O_6$: C, 68.1; H, 4.5%.

Upon admixture with isohibalactone, m.p. 156°C, the melting point depressed to 140～2°C. The infrared absorption spectrum and ultraviolet spectrum of this lactone agreed completely with authentic (−) hibalactone

(±)-Hibalactonic acid.

(±)-Hibalactone (1.0 g) was boiled with 10 ml of 1 N-NaOH and 20 ml of ethanol for 10 minutes, and the clear solution diluted with water. On cooling the sparingly soluble sodium salt precipitated as colorless leaflets, m.p. 108～9°C. The mixture was chilled in ice, and acidified with acetic acid. The product (0.9 g) which was collected after several hours and dried in vacuo, formed colorless felt-like needles, m.p. 143～4°C. *Anal.* Found: C, 64.99; H, 4.94. Calcd. for $C_{20}H_{18}O_7$: C, 64.9; H, 4.8%.

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On the Metabolism of Organic Acids by *Clostridium acetobutylicum*

Part I. Formation of Lactic Acid and Racemiase

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Authors confirmatively observed the phenomena that in adequate conditions, the mode of acetone-butanol fermentation could be converted into homo-lactic acid fermentation, and that the formation of "Racemiase" (lactic acid racemiase) was intensified through the appropriate lactate level in growing culture of the bacterium, *Clostridium acetobutylicum*.

Furthermore in this series of the papers, the possible role of "Racemiase" in the lactic acid metabolizing sequence was suggested, and the chemical properties of "Racemiase" as well as the mechanism of enzymatic racemization of lactic acid were investigated. The metabolism of several organic acids were also discussed in connection with the fermentation pathway of this organism.

INTRODUCTION

In the carbohydrate metabolism of acetone-butanol fermentation bacterium, *Clostridium acetobutylicum*, organic acids such as pyruvic, acetic, formic, butyric and lactic acids are detected as the intermediary or end products of the regular fermentation. It is regarded as the fixed concept that the Embden-Meyerhof-Parnas pathway is found in the fermentation of *Cl. acetobutylicum* and that pyruvic acid, an intermediate in the metabolic sequence, is cleaved to carbon dioxide and to "acetyl" unit, the latter is further subjected to the condensation or the reduction in forming various fermentation products. Considerable amounts of acetic and butyric acids are ordinarily found in the fermented culture. Lactic acid together with formic acid are regarded as the minor products in normal metabolic situation, although the alkaline reaction of culture medium enhances the accumulation of these acids.

The formation of lactic acid by the acetone-butanol fermentation bacterium was previously found by Speakman in 1923[1]. Osburn et al.[2] observed the formation of 0.6 mole lactic acid per mole glucose consumed from the glucose-bicarbonate culture of *Cl. butylicum*. Furthermore, Brown et al.[3] verified the incorporation of isotopic bicarbonate into the carboxyl carbon of lactic acid with the fermentation of glucose by *Cl. butylicum*, and suggested that lactic acid would be derived from acetic acid and carbon dioxide. On the other hand, the tendency of the change of normal butanol fermentation into homo-lactic acid fermentation was found with several species of genus *Clostridium*. For instance, Kempner[4], Kempner and Kubowitz[5] and Kubowitz[6] described that the butyric acid fermentation of *Cl. butyricum* was compelled to convert its fermentation into homo-lactic acid fermentation by carbon monoxide, cyanide, dipyridyl or hydrogen. With iron-deficient *Cl. welchii*, Pappenheimer and Shaskan[7] reported a shift of butanol fermentation into lactic acid fermentation. The same results were obtained with iron-deficient *Cl. perfringens* by Bard and

1) H.B. Speakman, *J. Biol. Chem.*, **58**, 395 (1923).
2) O.L. Osburn, R.W. Brown and C.H. Werkman, *J. Biol. Chem.*, **121**, 685 (1937).
3) R.W. Brown, H.G. Wood and C.H. Werkman, *Arch. Biochem.*, **5**. 423 (1945).
4) W. Kempner, *Biochem. Z.*, **257**, 41 (1933).
5) W. Kempner and F. Kubowitz, *Biochem. Z.*, **265**, 245 (1933).
6) F. Kubowitz, *Biochem. Z.*, **274**, 285 (1934).
7) A.M. Jr. Pappenheimer and E. Shaskan, *J. Biol. Chem.*, **155**. 265 (1944).

Gunsalus[8]. Employing cell-suspensions of *Cl. acetobutylicum*, Simon[9] obtained 1.36 g of racemic zinc lactate from 3 g of glucose in the presence of atmospheric carbon monoxide. In the present paper, the results of various experiments, that a strain of *Cl. acetobutylicum* accumulated lactic acid, are described.

With the racemic lactic acid producing *Lactobacilli*, Katagiri and Kitahara[10] postulated the enzymatic racemization of optically active lactic acid which was formed by the organism. To this characteristic enzyme, which mediated the racemization of optically active lactic acid, the term "Racemiase" was offered by them[11]. Subsequently such an enzymic action was demonstrated with *Cl. butylicum*[12,13], *Cl. acetobutylicum*[12,13,14] and *Staphylococcus ureae*[14]. The lactic acid bacteria concerning "Sake" brewing, produce originally optically active lactic acids in pure culture, but in natural fermentation, for instance in "Sake" brewing, lactic acid, found in broth, is always racemic. This characteristic phenomenon was expounded by Katagiri and Kitahara[15] that the optically active lactic acids produced by lactic acid bacteria were subsequently racemized through the action of racemiase of *Clostridium* or of inactive lactic acid producing bacteria. In fact, optically active lactic acid submitted to either growing culture of *Cl. acetobutylicum* or cell-suspensions of the same bacterium was found to be racemized[14]. Authors of the present paper, observed the inducing action of lactic acid on the formation of racemiase and the enzymatic racemization of lactic acid with *Cl. acetobutylicum*. Addition of lactic acid to the culture medium revealed remarkable acceleration of racemiase formation. Furthermore, it was shown that in adequate situation, in which the accumulation of lactic acid in bacterial culture was facilitated, the formation of racemiase was intensified.

8) R. C. Bard and I. C. Gunsalus, *J. Bacteriol.*, **59**, 387 (1950).
9) E. Simon, *Arch. Biochem.*, **13**, 237 (1947).
10) H. Katagiri and K. Kitahara, *J. Agr. Chem. Soc. Japan*, **12**, 281 (1936).
11) H. Katagiri and K. Kitahara, *J. Agr. Chem. Soc. Japan*, **12**, 844 (1936).
12) E.L. Tatum, W.H. Peterson and E.B. Fred, *Biochem. J.*, **30**, 1892 (1936).
13) W. B. Christensen, M. J. Johnson and W. H. Peterson, *J. Biol. Chem.*, **127**, 421 (1939).
14) H. Katagiri and K. Kitahara, *J. Agr. Chem. Soc. Japan*, **12**, 1217 (1936).
15) H. Katagiri and K. Kitahara, *Biochem. J.*, **31**, 909 (1937).

MATERIALS AND METHODS

Microorganism and culture

A strain of *Clostridium acetobutylicum* which was isolated in this laboratory was used throughout the investigations. The composition of basal medium was as follows[1]; glucose 2%, peptone 0.5%, monobasic potassium phosphate 0.05%, dibasic potassium phosphate 0.05%, magnesium sulphate 0.02%, sodium chloride 0.001%, manganous sulphate 0.001% and ferrous sulphate 0.001%. Ferrous sulphate was sterilized separately and added just before the inoculation. The microorganism was stocked on sand culture in the sealed tube. A small amount of the sand culture was inoculated into five per cent maize mash, heat-shocked for about sixty seconds in boiling water, kept in vacuum vessel and incubated at 37°C for two or three days. The maize mash culture was used as the inoculum for the culture on a large scale.

Preparation of the optically active lactic acids

The laevo-rotatory lactic acid (D(−)-lactic acid) was obtained from the culture broth of *Lactobacillus delbrückii* (D-lactic acid former) grown in a medium containing saccharified potato starch and malt extract, and purified by recrystallization as its zinc salt. In the same manner, *Streptococcus faecalis* (L-lactic acid former) was used for the preparation of dextro-rotatory lactic acid (L(+)-lactic acid).

Analysis of fermentation products

The amount of glucose was estimated by Bertrand's method. Butyl and ethyl alcohols were determined by the method of Johnson[16]. The analysis of acetone was carried out with Messinger's method modified by Godwin[17]. Both butyric and acetic acids were determined by Virtanen and Pulki's duplicate distillation method[18]. Pyruvate was determined by the modified Friedemann-Haugen's method[19], and the colorimetry of Barker and Summerson[20] was applied for the estimation of lactic acid.

16) M. J. Johnson, *Ind. Eng. Chem., Anal. Ed.*, **4**, 20 (1932).
17) L. F. Godwin, *J. Am. Chem. Soc.*, **42**, 39 (1920).
18) A. I. Virtanen and L. Pulki, *J. Am. Chem. Soc.*, **50**, 3138 (1928).
19) T. E. Friedemann and G. E. Haugen, *J. Biol. Chem.*, **147**, 415 (1943); T. Shimizu, *Seikagaku (Japan)*, **22**, 108 (1950).
20) S. B. Barker and W. H. Summerson, *J. Biol. Chem.* **138**, 535 (1941).

Assay of racemiase activity

Optically active lactic acid was submitted to the reaction mixture to final concentration 0.1 to 0.2 M, adjusted to pH 6.5 and incubated at 37°C for at least eight hours in the presence of few drops of toluene. When the reaction mixture contained intact cells, it was supplemented with one or two drops of chloroform. The resultant racemic acid was estimated by the optical rotatory power of the isolated lactic acid being extracted with ether from the reaction mixture. Occasionally, the amount of water of crystallization in the zinc salt of the isolated acid was determined, so as to confirm the experimental results obtained by polarimetry. The enzymatic determination of each one of the optical isomers of lactic acid, by the established method of Kitahara and Fukui[21], was conveniently applied to semi-micro and micro experiments of racemiase assay, because this method was facilitated in estimating separately an optical isomer of lactic acid in the amount of 1 to 5 μmoles, within an hour. In our case, the racemiase assays of small scale were performed by using L-lactic acid as the substrate, and the degree of racemization was estimated by determining the resultant D-lactic acid manometrically with the aid of dried cells of *Leuconostoc mesenteroides** as the enzyme preparation of D-lactic dehydrogenase system by the method of Kitahara and Fukui.

The paper chromatographic separation of zinc salts of both racemic and optically active lactic acids was established by authors. The details of this procedure are presented below.

Paper chromatographic separation of racemic and optically active lactic acid

Overcoming the inconvenience of ether extraction of lactic acid from the reaction mixture and of the observation of optical rotatory power of the extracted acid, authors made an attempt to distinguish between racemic and optically active lactic acids by means of paper partition chromatography. Both types of free lactic acid show the same solubility against miscellaneous solvents, but the metal salts of lactic acids show different characteristic properties. For instance, zinc salts of both racemic and optically active lactic acids include three and two molecules of water of crystallization respectively. Furthermore, these salts vary in solubility in water each other. Zinc salts of dextro- and laevo-rotatory lactic acids are soluble as much as 5.7 per cent in water at 15°C[22,23]. Racemic zinc lactate is, however, less soluble in water at the same temperature. Namely, one part of racemic zinc lactate is soluble in 58.7, 55.97, 53 and 6 parts of water at 8, 10, 15 and 100°C respectively[24,25,26,27]. Taking into consideration, the difference of the solubility in water, the polarizing properties of butyl and ethyl alcohols, and the influence of hydrogen ion concentration of the chromatographic localization of the electrolytes such as zinc lactate, authors carried out the paper partition chromatography of zinc lactate by various solvent systems. Consequently, the solvent system of the following composition was established; that is, the mixture of three parts of water-saturated butyl alcohol, two parts of ethyl alcohol and two parts of water, and adjusted to pH 6.4. Three per cent aqueous solution of zinc lactate, which was prepared from the ether-extracted lactic acid in the reaction mixture, was sampled on the filter paper as a band of few centimeters length. After developing for sixteen hours in ice box, the paper was dried and exposed to hydrogen sulphide in the closed vessel about thirty minutes so as to make the localized zinc as Znlactate to its sulphide. Zinc sulphide thus formed on paper chromatogram, after liberation of excess hydrogen sulphide, was detected as the black spot of cuprous sulphide by spraying 4 per cent aqueous solution of cupric sulphate. By this solvent system, zinc salts of optically active lactic acids were localized at the R_F value of 0.55, although that of racemic lactic acid remained at the origin. The mixture in various ratios of both types of zinc lactates could be separated each other qualitatively and fairly quantitatively. This method is convenient for the detection of racemized lactic acid in the reaction mixture of racemiase assays.

RESULTS AND DISCUSSION

Inducing effect of lactate on the formation of racemiase

Katagiri et al.[28] previously pointed out the inducing effect of lactate, which was supplemented in Speakman's culture medium, on the formation of racemiase. Additional results on this problem are shown in Table I. The results, shown in Table I, indicate that supplemented

21) K. Kitahara and S. Fukui, *J. Agr. Chem. Soc. Japan*, **26**, 555 (1952).

* *Leuconostoc mesenteroides* is a hetero-lactic fermenter, possesses D-lactic dehydrogenase and produces only D-isomer of lactic acid. Dried cells here used were harvested from malt extract culture.

22) J. Wislicenus, *Ann.*, **167**, 314 (1873).
23) G. Tate, *J. Chem. Soc.*, **63**, 1267 (1893).
24) H. L. Buff, *Ann.*, **140**, 160 (1866).
25) J. Wislicenus, *Ann.*, **126**, 228 (1863).
26) A. Strecker, *Ann.*, **105**, 316 (1858).
27) H. Engelhardt and R. Maddrell, *Ann.*, **63**, 103 (1847).
28) H. Katagiri, K. Imai, T. Tochikura and T. Nakamura, *Symposia on Enzyme Chemistry (Japan)*, **8**, 107 (1953).

TABLE I. EFFECT OF LACTIC ACID ON THE FORMATION OF RACEMIASE

Modification of culture medium			Glucose consumed	DL-Lactic acid		Racemiase activity*
Glucose (%)	Peptone (%)	Additions	(%)	Initial (mg%)	Final (mg%)	
2.0	1.0	——	61.5	0	11	13.1
2.0	——	Yeast extract**	20.0	0	57	15.6
2.0	1.0	DL-Lactic acid	100.0	930	153	73.9
——	1.0	DL-Lactic acid	——	1,860	1,820	0

Components of the basal medium were presented in the text.
* Degree of racemization (%) of L-lactic acid submitted as much as 0.5 g per 100 ml cultured solution.
** Boiled extract from 1 g of dried brewers' yeast was added to 100 ml of the culture medium.

TABLE II. SEVERAL ASPECTS OF CULTURE AND RACEMIASE FORMATION

Components except stated were similar to the basal medium. Seventy-two hours cultures were freed from cells and supplemented with 1 g of calcium carbonate as well as 1 g of D-lactic acid (Na-salt) per 200 ml. Adding few drops of chloroform and toluene, the reaction mixture was incubated at 37°C for 48 hours. Lactic acid in the reaction mixture was extracted and converted to zinc salt. The degree of racemization was estimated from paper chromatogram and contents of water of crystallization respectively.

Culture medium	I	II	III	IV	V	VI
Glucose (%)	2.0	0.5	4.0	2.0	2.0	2.0
Peptone (%)	0.5	1.0	0.5	0.5	0.5	1.0*
DL-Lactic acid (%)	0.15	0.15	0.15	0.15	0.15	0.15
Additions (except salts mixture)	none	none	none	none	10^{-4}M $FeSO_4$	Vitamin mixture**
Initial pH	6.4	6.4	6.4	7.6	6.4	6.4
Gas evolution						
24 hours	++	+++	++	±	+++	+++
48 hours	+++	+	+++	+	+	+++
After 72 hours						
Final pH	4.2	4.6	4.2	4.4	4.2	4.2
Residual sugar	nil	nil	nil	nil	nil	nil
Volatile acids (mg% as acetic)	768	388	848	224	760	900
Racemiase activity						
a) from PPC Detection of DL-lactate	+	+++	++	+++	−	++
Degree of racemization	20	90	40	90	0	40
b) from water of crystallization						
Degree of racemization	47.6	94.0	46.8	96.0	0	38.1

* Corresponding amount of peptone was hydrolyzed in 10% sulfuric acid under the pressure of 15 lbs. for 1 hour and was recovered from sulfuric acid.
** Ingredients and final concentrations were as follows: thiamine 200 γ%, Ca-pantothenate 200 γ%, nicotinic acid 100 γ%, pyridoxine 100 γ%, PABA 1 γ%, folic acid 1 γ% and biotin 1 γ%.

DL-lactate is easily consumed and is capable of inducing the formation of racemiase, although lactate can not be a sole carbon source for the growing of *Cl. acetobutylicum*, and that peptone is essential for the both growth and racemiase formation of this organism in Speakman's culture medium.

Investigation on the cultural conditions for the formation of racemiase

Several aspects of the fermentation and the formation of racemiase under various cultural conditions are presented in Table II. From the results shown in Table II, the followings would be inferred as the affecting factors on the

Table III. Proportion of Peptone to Glucose on the Formation of Racemiase

Each run was supplemented with 0.15% lactic acid. Forty-eight hours culture was added with $CaCO_3$, Na-L-lactate, chloroform and toluene. Reaction time was 48 hours. The degree of racemization was estimated manometrically.

Culture medium		L(+)-Lactic acid used (γ/ml)	DL-Lactic acid formed (γ/ml)	Degree of racemization (%)
Glucose (%)	Peptone (%)			
4.0	1.0	4500	2048	45.5
2.0	1.0	4500	1824	40.5
1.0	1.0	4500	1600	35.5
0.5	1.0	4500	944	20.9
4.0	0.5	4500	1184	26.3
2.0	0.5	4500	1120	25.3
1.0	0.5	4500	864	19.2
0.5	0.5	4500	720	16.0

Table IV. Effects of Lactic Acid and Calcium Carbonate on the Formation of Racemiase (I).

Peptone content of the basal medium was modified to 1%. Forty hours culture was submitted to the racemiase assay.

Additions to culture		Glucose consumed	DL-Lactic acid		Racemiase activity*
DL-lactic acid (%)	Calcium carbonate (%)	(%)	Initial (mg%)	Final (mg%)	
0.15	5.0	87.5	163	210	81
0.15	0	61.0	163	18	83
0	5.0	70.0	0	108	90
0	0	51.5	0	14	29

* Degree of racemization (%) of L-lactic acid, submitted as much as 0.5 g per 100 ml cultured solution.

Table V. Effects of Lactic Acid and Calcium Carbonate on the Formation of Racemiase (II).

Experimental conditions were similar to Table IV.

Additions to culture		Final pH	Glucose consumed (%)	Racemiase activity*
DL-Lactic acid (%)	Calcium carbonate (%)			
0.15	0	4.4	40.6	59
0.15	5.0	5.2	57.6	63

* Degree of racemization (%) of L-lactic acid, submitted as much as 0.5g per 100 ml cultured solution.

formation of racemiase: i) The proportion of peptone to glucose, ii) the additional amount of ferrous ion, iii) the initial pH value, iv) accumulation of volatile acids in the culture broth, and v) character of the organic nitrogen source. Dealing with the proportion of peptone to glucose, the results were obtained as shown in Table III. Throughout the experiments, the amount of additional lactic acid was fixed as 0.15 per cent in conformity with the previous report[28]. As shown in Table III, the quantity of peptone in the basal medium was modified to one per cent.

Effect of calcium carbonate on the formation of racemiase

The normal fermentation of *Cl. acetobutylicum* proceeds a characteristic time course with respect to acid formation. In early stage of the

fermentation, the remarkable increase of the acidity of culture medium can be observed. This acid-increase phase is then alternated with the acid-decrease phase, in which the acidity is rapidly dropped and, on the other hand, the amount of neutral solvents such as butyl and ethyl alcohols and acetone are increased. Accordingly the accumulation of acids could be expected by preventing the shift of these acids to neutral solvents in some ways. In other words, adequate acid-trapping agents might convert the normal fermentation into acid forming type. In this connection, calcium carbonate was employed in the culture of this organism, and as a result of it, the increase of lactic acid formation was detected in the medium and the remarkable acceleration of racemiase formation was revealed. The experimental results were shown in Table IV. These results suggest that calcium carbonate is capable of taking the place of lactic acid in the induction of racemiase formation by *Cl. acetobutylicum*. As seen in Tables IV and V, it is without doubt that pre-existing lactic acid shows predominant effect on racemiase formation irrespective of supplemented calcium carbonate.

Time course of the formation of lactic acid and racemiase

From the results obtained in the experiments of lactate supplemented culture as well as calcium carbonate added culture, it would be presumable that the adequate amount of lactic acid in the culture medium may play a role in the formation of racemiase by *Cl. acetobutylicum*. It means that lactic acid, though it may be supplemented or produced by the organism, is essential as an inductor to form racemiase. Accordingly, the relationship between the level of lactic acid in the culture medium and the time course of racemiase formation will be noticeable in discussing the problem of inductor and inducing enzyme. Figs. 1 to 3 demonstrate typical time course curves of fermentation, level of lactic acid and racemiase activity in the culture medium in several cultural conditions. Ferrous ion is regarded as a cofactor of aldolase[8], phosphoroclastic system of pyruvate[29], and hydrogenase[30] of *Cl. acetobutylicum* or related bacteria. In this connection, the influence of additional ferrous sulphate (Fig. 2) was investigated. As shown in Fig. 2, ferrous ion promoted the consumption of sugar but depressed the formation of lactic acid. In comparison with Fig. 2, the case of excess amount of ferrous ion was present, fluctuation of the level of lactic acid was observed in normal and lactate added fermentations as shown in Figs. 1 and 3. Ac-

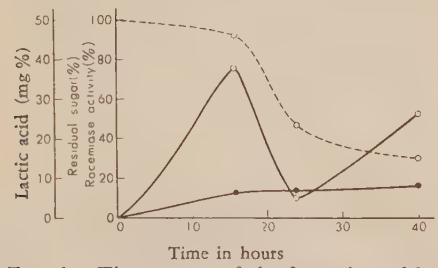

FIG. 1. Time course of the formation of lactic acid and racemiase by *Cl. acetobutylicum* in Speakman's medium, containing 2% glucose, 1% peptone, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% NaCl, 0.001% $MnSO_4 \cdot H_2O$ and 0.001% $FeSO_4 \cdot 7H_2O$

----○---- Residual sugar (%)
——○—— Lactic acid (mg %)
——●—— Racemiase activity (%)

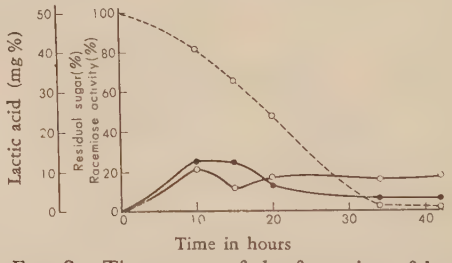

FIG. 2. Time course of the formation of lactic acid and racemiase by *Cl. acetobutylicum* in iron-enriched Speakman's medium. The medium was supplemented with ferrous sulphate to final concentration 1×10^{-4} M (abont 0.003%). Other ingredients were similar to Fig. 1.

----○---- Residual sugar (%)
——○—— Lactic acid (mg %)
——●—— Racemiase activity (%)

29) R. S. Wolfe and D. J. O'Kane, *J. Biol. Chem.*, **205**, 755 (1953).
30) H. D. Jr. Peck and H. Gest, *J. Bacteriol.*, **73**, 569 (1957).

companying with the fluctuation of lactate level in culture medium, the variety of racemiase activity appeared. As a rule, the ascent of lactate level intensified the formation of racemiase, and the racemiase activity seemed to be maintained by comparatively high levels of lactate in the culture. Even in the case of lactate added culture, the increased amount of lactic acid could be detected. Lactic acid once accumulated was suddenly consumed by the organism except for the case in which appropriate amount of lactic acid was not formed because of the presence of excess amount of ferrous ion. From the results in Fig. 3, it is assumable that the initial level of lactic acid is essential for the formation of racemiase, and that the increase of lactic acid in early stage of fermentation and the following rapid consumption of it may play a significant role in intensifying the activity of racemiase in culture medium. In other words, the racemiase activity could be obtained in such a culture in which

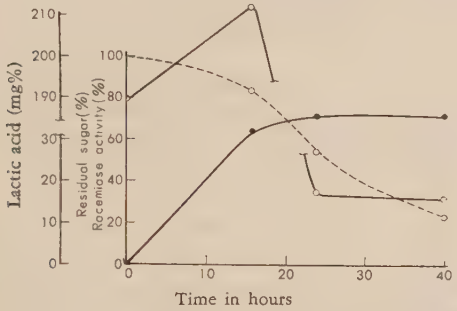

FIG. 3. Time course of the formation of lactic acid and racemiase by *Cl. acetobutylicum* in lactate-added Speakman's medium. The medium was supplemented with DL-lactic acid sodium salt to final concentration 2×10^{-2}M (about 0.18% as free acid). Other ingredients were similar to Fig. 1.

—○---- Residual sugar (%)
—○— Lactic acid (mg %)
—●— Racemiase activity (%)

TABLE VI. EFFECTS OF PYRUVATE AND FERROUS ION ON THE FORMATION OF LACTATE AND RACEMIASE

Basal medium contained 2% glucose, 1% peptone, 0.05% K_2HPO_4, 0.05% KH_2PO_4, 0.02% $MgSO_4\cdot7H_4O$, 0.001% NaCl and 0.001% $MnSO_4\cdot H_2O$. In iron-deficient culture, Exp. I and III, critical amount of iron seems to be supplied from the ingredients of basal medium.

Experiment	Culture time (hours)	0	20	40
I DL-Lactate added	pH	6.4	4.6	4.6
	Glucose consumed (%)	—	60.2	97.4
	Lactic acid (mg%)	222.0	13.4	4.3
	Pyruvic acid (mg%)	0	11.2	0.8
	Racemiase activity*	—	88.5	37.0
II DL-Lactate and iron added (1.4×10^{-4} M $FeSO_4\cdot7H_2O$)	pH	6.4	4.4	4.4
	Glucose consumed (%)	—	84.0	96.4
	Lactic acid (mg %)	222.0	10.4	1.7
	Pyruvic acid (mg%)	0	11.6	1.0
	Racemiase activity*	—	34.6	4.9
III Pyruvate added	pH	6.4	4.6	4.6
	Glucose consumed (%)	—	64.7	98.5
	Lactic acid (mg%)	0	66.0	12.8
	Pyruvic acid (mg%)	93.0	7.5	2.9
	Racemiase activity*	—	40.7	18.1
VI Pyruvate and iron added (1.4×10^{-4}M $FeSO_4\cdot7H_2O$)	pH	6.4	4.6	4.6
	Glucose consumed (%)	—	90.1	98.2
	Lactic acid (mg%)	0	16.2	9.2
	Pyruvic acid (mg%)	93.0	16.2	1.7
	Racemiase activity*	—	25.4	0

* See Table V.

TABLE VII. EFFECT OF CYANIDE ON THE FORMATION OF LACTATE AND RACEMIASE

To the basal medium added potassium cyanide to final concentration 5×10^{-3}M. Forty-five mg L-lactic acid was submitte to 5 ml culture filtrate together with 0.5 g $CaCO_3$ and 1 drop of toluene, and the mixture was incubated at 37°C for 17 hours. Degree of racemization was estimated manometrically.

Culture time (hrs)	Control				DL-Lactate added				
	Glucose consumed (mg%)	Degree of fermentation (%)	Lactic acid (mg%)	Racemiase activity	Glucose consumed (mg%)	Degree of fermentation (%)	Lactic acid (mg%)	Racemiase activities	
0	—	—	0	—	—	—	112.5	—	—
10	19.9	1.0	15.0	0	23.9	1.2	86.0	1.6	—
15	209.0	10.5	17.2	—	189.1	9.5	99.0	—	—
20	238.8	12.0	16.8	2.0	248.8	12.5	114.0	6.0	—
34	559.2	28.1	93.6	—	439.8	22.1	115.0	—	—
42	559.2	28.1	145.0	0	449.7	22.6	160.0	0.5	18.7*

* Racemiase activity estimated with fivefold concentrated culture filtrate.

lactic acid is metabolized vigorously at relatively high level of its amount. Accordingly, the relationship between the metabolism of lactic acid and the physiological role of racemiase in this organism can be assumed. In connection with this problem, further investigations and discussion will be presented in subsequent paper.

Effects of pyruvate and ferrous ion on the formation of lactate and racemiase

The mechanism of the formation of lactic acid by acetone-butanol fermentation bacterium is considered that pyruvic acid, formed through the glycolysis, is reduced to lactic acid in coupling reaction with the dehydrogenation of triose phosphate[9]. Meanwhile, in normal fermentation, pyruvate is further degraded in forming acetyl compound by phosphoroclastic enzyme system, in which ferrous ion is considered to play a catalytic role[29]. In this connection, the effects of pyruvic acid on the formation of lactic acid and racemiase both in iron-sufficient and deficient culture media were compared with those in the presence of supplemented lactic acid. The results were shown in Table VI. In iron-deficient culture, the addition of pyruvic acid promoted temporarily the accumulation of lactic acid and intensified the formation of racemiase. Sufficient amount of iron, however, restrained such a trend. On the whole, racemiase activities were distinctly suppressed in iron-sufficient culture inspite of the vigorous fermentation.

Effect of potassium cyanide on the formation of lactate and racemiase

As a means of blocking the degradation of pyruvic acid, potassium cyanide was added to the culture medium, and the amounts of lactic acid as well as racemiase activities were pursued. Though it was not asserted that the initial concentration of cyanide could be maintained throughout the experiments because of the slightly acidulated reaction of culture media of pH 6 to 7, the accumulation of lactic acid was remarkably enhanced by the addition of cyanide as is shown in Table VII. Both the depression of fermentation and the accumulation of lactic acid could be considered to be derived from blocking metalloaldolase and pyruvate degrading enzyme. Though cyanide is a potent inhibitor of racemiase (see the subsequent paper), the significant activity of racemiase was detected in concentrated culture solution which had been supplemented with cyanide as indicated in Table VII, and lactic acid accumulated in culture was mostly optical-inactive. It should be further investigated whether initially formed optically active lactic acid was to be inactivated by significant amount of racemiase withstanding the supplemented cyanide.

It is shown in Table VIII that cyanide prolonged culture times of *Cl. acetobutylicum* and

Table VIII. Formation of Lactic Acid in Cyanide Added Culture

To the basal medium added potassium cyanide to final concentration 1×10^{-3} M.

Culture time (days)	1	5	13
Glucose consumed (mg%)	31	180	1850
Degree of fermentation (%)	1.6	9.7	100.0
Lactic acid (mg%)	20	103	1020
Lactic acid/Glucose (mole ratio)	1.33	1.14	1.10

offered a shift of its fermentation to form lactic acid.

Effect of sodium bicarbonate on the formation of lactate and racemiase

Brown et al.[3] pointed out the incorporation of submitted carbon dioxide into carboxyl group of lactic acid by *Cl. butyricum* grown in glucose medium. With *Cl. acetobutylicum*, the addition of bicarbonate to Speakman's medium promoted the formation of lactic acid as shown in Table IX. It is still doubtful whether the accumulated lactic acid by the addition of bicarbonate was originated in the fixation of carbon dioxide or

Table IX. Effect of Sodium Bicarbonate on the Formation of Lactate and Racemiase

To the basal medium added 100 mg% of $NaHCO_3$ after 16 hours incubation in Experiment B.

	Culture time (hours)	0	16	24
Experiment A	pH	6.4	4.4	4.2
	Glucose (mg%)	1590	610	540
	Lactic acid (mg%)	15.2	10.7	7.3
	Pyruvic acid (mg%)	0.8	4.5	5.6
	Racemiase activity*	—	66.7	87.2
Experiment B	pH	6.4	4.4	8.8
	Glucose (mg%)	1670	690	590
	Lactic acid (mg%)	18.4	11.7	40.7
	Pyruvic acid (mg%)	0.8	3.5	37.0
	Racemiase activity*	—	65.8	18.3

* Forty-five mg of L-lactic acid was submitted to 5 ml culture filtrate together with 0.5 g $CaCO_3$ and 1 drop of toluene, and the mixture was incubated at 37°C for 17 hours. The degree of racemization (%) was estimated manometrically.

in the maintenance of relatively high pH range owing to the buffering action of bicarbonate as in the case, in which the neutral reaction of medium is sustained by phosphate buffer (see the next article). At any rate, remarkable increase in amounts of lactate and pyruvate was resulted from bicarbonate supplemented. In Experiment B in Table IX, the consumption of glucose between sixteen to twenty-four hours was 100.0 mg, and the increments of lactic and pyruvic acids during this period were 39.0 and 33.5 mg respectively. These data suggest that the bulk of glucose consumed was converted to both acids in consequence of the rise of pH value of culture medium. The contrary depression of racemiase activity was considered to be due to the inhibitory effect of alkaline reaction of the medium.

Fermentation of glucose and the formation of lactate by intact cells of *Clostridium acetobutylicum*

As described above, cyanide is capable of accumulating lactic acid in glucose culture medium. Cells of *Cl. acetobutylicum* grown in Speakman's medium containing 0.15 per cent lactic acid converted more than eighty per cent of glucose consumed to lactic acid by the use of cyanide 0.01 M at pH 6.4. Even in the absence of cyanide, these cell-suspensions formed significant amount of lactic acid as shown in Table X. Typical

Table X. Fermentation of Glucose by Intact Cells of *Clostridium acetobutylium*

Cells were obtained from 20 hours culture in the basal medium supplemented with 0.15% lactic acid, washed by water and stored overnight in ice box. Cells from 1 liter of the culture were submitted to the medium containing 3.5% glucose and Speakman's salts mixture (similar to the basal medium) to final volume 20 ml, and pH was adjusted to 6.4. Incubation was durated for 72 hours at 37°C. In Experiment B, potassium cyanide was added for molar concentration 0.01 at the start.

Substrate and products (mg%)	Experiment A	Experiment B
Glucose (initial)	3470.0	3470.0
Glucose (consumed)	1660.0	820.0
Butyl alcohol	269.0	28.5
Ethyl alcohol	18.5	6.2
Acetone	45.4	19.3
Butyric acid	48.2	1.8
Acetic acid	44.6	48.4
Lactic acid	455.0	682.0
Lactic acid/Glucose consumed (%)	27.4	83.2

TABLE XI. FORMATION OF LACTIC ACID BY INTACT CELLS of *Clostridium acetobutylicum*

Cells were obtained from 70 hours culture in the basal medium with no ferrous sulphate. Incubation mixture was composed of washed cells from 500 ml culture, 1 g glucose and 1 m mole phosphate buffer (pH 7.2) to final volume 20ml, and incubated for 96 hours at 37°C. In Experiment B, potassium cyanide was added for molar concentration 0.01 at the start.

	Experiment A	Experiment B
Glucose consumed (mg%)	1280	910
(%)	35.8	25.5
Lactic acid formed (mg%)	1241	736
D-(−)-lactic acid (mg%)*	607	334
DL-lactic acid (mg%)**	1214	668
Ratio of lactic acid formed to glucose consumed (%)	97.0	81.0

* Determined by the method of Kitahara and Fukui[28].
** Duplicate value of D-lactic acid.

homolactic acid fermentation of glucose by cells of this organism was performed in 0.05 M phosphate of pH 7.2. Results were inserted in Table XI. In this occasion, cyanide seemed to be dispensable for the shift of fermentation to homolactic form, and the high concentration of phosphate above pH 7 was rather available for the conversion of glucose into lactic acid. Moreover, lactic acid thus obtained was found to be almost racemic from manometric assay by Kitahara and Fukui's method, namely, duplicate of D-lactic acid which was estimated by manometric technique was nearly equal to total amount of lactic acid determined colorimetrically.

Inclusively, lactic acid, which, under normal conditions, is not a metabolic product of this organism, is accumulated by blocking the cleavage of pyruvic acid to acetyl compound. As the mechanism of lactic acid formation, the pathway of homo-lactic acid fermentation is most probable, in which diphosphopyridine nucleotide transports hydrogen from triose phosphate to pyruvate in forming lactic acid.

SUMMARY

The inducing action of lactic acid on the formation of racemiase was investigated in various aspects. Calcium carbonate revealed remarkable effect on the formation of racemiase, when lactate was not added as an inductor. Characteristic time course of formation of both lactic acid and racemiase in culture of *Cl. acetobutylicum* was pursued. Pyruvate was capable or promoting racemiase activity to some extent in iron-deficient culture. Lactic acid was produced extraordinarily in the presence of cyanide of bicarbonate, but the formation of racemiase was not affected significantly. With intact cells or *Cl. acetobutylicum* in adequate conditions, homo-lactic acid fermentation was realized, namely, ninety-seven per cent of glucose consumed was converted to racemic lactic acid. Details of paper chromatographic technique in separating racemic and optically active zinc lactates were presented.

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2, p. 173~181, 1960]

On the Metabolism of Organic Acids by *Clostridium acetobutylicum*

Part II. Lactic Acid Metabolism and Relating Role of Racemiase

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With racemiase free bacterial cells of *Clostridium acetobutylicum*, distinguishable mode of the metabolism of both D- and L-lactic acids was found. Racemiase rich cells, however, metabolized both types of lactic acids to a same extent. It was indicated that this organism originally possessed D-lactic dehydrogenase, and anaerobically dehydrogenated D-lactic acid, and that the organism oxidized aerobically L-lactic acid by a lactic acid oxidizing enzyme which was proposed in this paper.

INTRODUCTION

Lactic acid, a minor product in normal culture of *Clostridium acetobutylicum*, is accumulated in adequate conditions as much as eighty to ninety per cent of glucose consumed. Moreover, lactic acid supplied to the growing culture of this organism is thoroughly fermented together with glucose. Not only under anaerobic circumstances, but also in the presence of air oxygen, *Cl. acetobutylicum* is capable of metabolizing lactic acid to some extent.

In the previous paper[1] the formation of lactic acid by *Cl. acetobutylicum* was discussed in several aspects. In all cases, in which lactic acid was formed, racemiase activity was detected in some measure, and lactic acid obtained was always optically inactive. In this investigation, the resolution of characteristics of lactic dehydrogenase in this organism was carried out, and finally indicated the existence of D-type lactic dehydrogenase in this organism.

As previously described, lactic acid added culture showed potent acceleration of racemiase formation, besides, almost whole amount of lactic acid supplemented to the culture medium disappeared during the fermentation. In this connection, it seems interesting to compare the metabolic sequence of D- and L-type lactic acids in relation to racemiase activity. It was revealed that *Cl. acetobutylicum* possessed D-lactic dehydrogenase and L-lactic oxidizing system, and the former was capable of transporting hydrogen from D-lactic acid to atmospheric oxygen via methylene blue as a potent carrier, meanwhile, the latter operated aerobically upon L-lactic acid in forming acetic acid and carbon dioxide without any intermediary carriers supplemented. In the presence of sufficient amount of racemiase, however, such a distinction of action of the organism on both types of lactic acid was vanished. Finally it was concluded that racemiase, in the metabolism of lactate by *Cl. acetobutylicum*, would play a role in connecting both D-lactic dehydrogenase and L-lactic oxidizing enzyme system.

1) H. Katagiri, K. Imai and T. Sugimori, *This Bulletin*, **24**, 163 (1960).

MATERIALS AND METHODS

Microorganism and culture.

A strain of *Clostridium acetobutylicum*[1] was used. According to the modification in Part I, the following composition of basal medium was employed; glucose 2%, peptone 1%, monobasic potassium phosphate 0.05%, dibasic potassium phosphate 0.05%, magnesium sulphate 0.02%, sodium chloride 0.001%, manganous sulphate 0.001% and ferrous sulphate 0.001%. Others remained the same as stated in the previous paper unless indicated.

TABLE I. INTRODUCTION OF LACTIC ACID INTO GLUCOSE FERMENTATION OF *Clostridium acetobutylicum*

Experiment No.	I	II	III	IV	V
Initial Glucose (mg%)	1249.0	1040.6	625.0	144.0	0
DL-Lactic acid (mg%)	0	327.5	982.5	1637.5	1965.0
Glucose consumed (mg%)	1249.0	1040.6	625.0	144.0	no growth
Products (mg%)					
Butyl alcohol	252.0	193.2	28.1	7.0	
Ethyl alcohol	61.5	40.7	131.9	56.4	
Acetone	78.2	21.3	15.0	7.3	
Butyric acid	44.5	263.5	125.0	41.6	
Acetic acid	149.1	142.5	78.3	60.7	
Lactic acid	3.7	48.8	966.0	1472.0	

TABLE II. EFFECT OF METHYLENE BLUE ON THE FORMATION OF LACTIC ACID IN GLUCOSE FERMENTATION BY *Clostridium acetobutylicum*

Experiment No.	I	II	III	IV
Additions and products (mg%)				
Initial Glucose	520.0	520.0	520.0	520.0
Lactic acid	0	0	1275.0	1275.0
Methylene blue	0	16.0	0	16.0
pH	6.4	6.4	6.4	6.4
Final pH	4.4	4.4	6.8	6.0
Glucose consumed	520.0	520.0	520.0	520.0
Butyl alcohol	31.8	29.8	106.6	30.2
Ethyl alcohol	15.2	14.0	35.8	20.4
Acetone	12.6	10.6	18.4	21.2
Butyric acid	187.2	193.8	190.6	14.4
Acetic acid	96.4	101.4	197.4	114.8
Pyruvic acid	1.0	1.2	7.8	6.4
Lactic acid	104.0	168.0	832.0	1450.0
Increase of lactic acid	104.0	168.0	−443.0	175.0

Analysis of fermentation products and assay of racemiase activity.

Each products in cultured solution and in reaction mixture of cell-suspensions were analysed by the methods similar to those described in Part 1. To assay racemiase activity, employed the manometric methods by Kitahara and Fukui[1].

Assay of lactic dehydrogenase.

Dehydrogenation of lactic acid was estimated in evacuated Thunberg-Borsook's tube using methylene blue as a hydrogen acceptor. Aerobically, amount of oxygen uptake was measured in Warburg's manometer with the reaction mixture similar to Thunberg-Borsook's method; that is, the dehydrogenation of lactic acid was translated into oxygen uptake, the latter was corresponding to reoxidation of reduced methylene blue, which accepted hydrogen from lactic dehydrogenase system.

RESULTS AND DISCUSSION

Introduction of lactic acid into glucose fermentation.

Glucose fermentation supplemented with lactic acid proceeded in some variations of the products. In this case, initially subjected lactic acid was rapidly decreased accompanying with the metabolism of glucose, and complete consumption of glucose was acquired. On the other hand, no growth could be observed in a medium employing DL-lactic acid as a sole carbon source. Table I represents the introduction of lactic acid into glucose fermentation of *Cl. acetobutylicum*. From the results in Table I, it is evident that a large quantity of lactic acid added to

glucose fermentation was consumed incompletely despite of complete disappearance of glucose, and both butyric acid and ethyl alcohol were increased in their rate to glucose consumed, while the rate of butyl alcohol was depressed. On the whole, the rate of two-carbon compounds were remarkably increased with addition of lactic acid to glucose fermentation of this organism. These results may suggest the decomposition of added lactic acid to two-carbon intermediate, common precursor of acetic acid and ethyl alcohol in any way.

Effect of methylene blue on the formation of lactic acid in glucose fermentation.

As a probable mechanism of the formation of lactic acid from glucose by *Cl. acetobutylicum*, the reduction of pyruvic acid accumulated by blocking further degradation was presented in the previous paper. It was further revealed that methylene blue added to glucose fermentation of this organism was capable of promoting lactic acid formation, moreover, preventing normal fermentation of both glucose and added lactic acid. The results were shown in Table II. In these experiments, supplemented methylene blue was converted to leuco-form (reduced form) in early stage of fermentation and maintained colorless state at least for forty hours thereafter. After forty hours the fermented broth recovered blue color of methylene blue (oxidized form) just by heating. These observations indicate that the reduction systems of this organism can easily reduce methylene blue. In other words, hydrogen generating sites, such as triose phosphate dehydrogenase and pyruvic acid splitting system, are capable of transferring hydrogen to methylene blue as a substitute for hydrogenase[2] and many other reducing enzymes, in which butyl and ethyl alcohol-forming enzymes[3,4] and lactic dehydrogenase are included. In this point of view, it will be expected that the accumulation of hydrogen by methylene blue protects the liberation of molecular hydrogen but accelerates the reducing enzyme in some extent. The data in Table II indicate predominant effect of methylene blue merely on the accumulation of lactic acid. Hydrogen atoms transferred to hydrogenase are destined to be liberated as hydrogen gas from the medium. Two reasons are adopted for the accumulation of lactic acid by methylene blue. One of them is the block of pyruvate degrading enzyme, and the other is the preferential transfer of hydrogen to lactic dehydrogenase for reducing pyruvic acid.

In acetone-butanol fermentation the redox potentials at pH 7 of various reducing systems are as follows[5]; diphosphopyridine nucleotide –320 mV, four-carbon compounds reducing system –280 to –290 mV, ethyl alcohol forming system –190 to –200 mV and lactic acid forming system –175 mV. Since the redox potential of methylene blue is about +10 mV, it is expected to draw the potential of medium near zero by supplementing with methylene blue. Such an ascent of redox potential will cause disadvantage for the reducing enzymes of lower redox potential, for instance four-carbon compounds forming system. If the redox potential of the medium were kept about –175 to –190 mV, reducing system of higher potential than that of the e.g. lactic acid forming system of –175 mV, would function preferentially. Accumulation of lactic acid would consequently occur. Utilization of leuco-methylene blue as a hydrogen donor in lactic acid formation, however, would not be probable, because the redox potential of methylene blue is much higher than that of lactic acid forming system. In other words, it is difficult to explain such an effect of methylene blue on the formation of lactic acid with the aid of the operation of lactic dehydrogenase of redox potential of –175 mV or so. In this connection, it is presumable that a new lactic acid forming system of redox potential higher than that of methylene blue might be occupied in

2) H. D. Jr. Peck and H. Gest, *J. Bacteriol.*, **73**, 569 (1957).
3) R. M. Burton and E. R. Stadtman, *J. Biol. Chem.*, **202**, 873 (1953).
4) H. G. Wood, R. W. Brown and C. H. Werkman, *Arch. Biochem.*, **6**, 243 (1945).
5) M. Hongo, *J. Agr. Chem. Soc. Japan*, **32**, A113 (1958).

this organism.

Lactic dehydrogenase of *Cl. acetobutylicum*.

Intact cells of *Cl. acetobutylicum* obtained from basal medium as well as 0.15 per cent lactic acid added medium were used as a source of lactic dehydrogenase. Results of assays in Thunberg-Borsook's tube arein dicated in Table III and Table IV. With glucose-grown cells the

TABLE III. LACTIC DEHYDOGENASE OF *Cl. acetobutylicum* (I) (glucose-grown cells)

Reaction mixture contained cell-suspension 0.5 ml (dry matter 62.5 mg), 0.2 M phosphate buffer (pH 7.2) 0.5 ml, 0.02 % methylene blue 0.5 ml and Na-lactate 0.5 ml (18 mg as free acid) to final volume 2.0 ml. Thunberg-Borsook's tube was evacuated and incubated at 37°C.

Substrate	None	D(−)-Lactate	L(+)-Lactate
Decoloration time (minutes)	>180	21	70

TABLE IV. LACTIC DEHYDROGENASE OF *Cl. acetobutylicum* (II) (Glucose-DL-lactate-grown cells)

Reaction mixture contained cell-suspension 0.5 ml (dry matter 71.5mg), 0.2 M phosphate buffer (pH 7.2) 0.5ml, 0.02 % methylene blue 0.5 ml and Na-lactate 0.5 ml (18 mg as free acid) to final volume 2.0 ml. Thunberg-Borsook's tube was evacuated and incubated at 37°C.

Substrate	None	D(−)-Lactate	L(+)-Lactate
Decoloration time (minutes)	>60	13	13

dehydrogenation of D(−)-lactic acid proceeded far rapidly than that of L(+)-lactic acid. Lactic dehydrogenase activity of glucose-DL-lactate-grown cells, meanwhile, was higher than that of glucose-grown cells and no appreciable difference in dehydrogenation velocities between D- and L-lactic acids was observed. The same facts were ascertained in manometric investigations as shownin Figs. 1 and 2. On the occasion in Fig. 1, when glucose-grown racemiase-deficient cells were used, the dehydrogenation velocity of L-lactic acid was below the half of D-lactic acid. Glucose-lactate-grown racemiase-rich cells, however, dehydrogenated not only D-lactic acid in much higher velocity but also L-lactic acid to

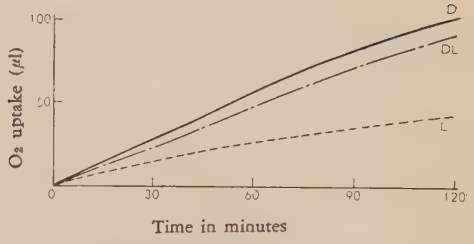


FIG. 1. Dehydrogenation of Lactic Acid by *Cl. acetobutylicum* grown in Speakman's Culture Medium.

Each Warburg vessel contained cell-suspension 1.0 ml (35mg dried cells, racemiase activity 6.3 %), 0.2 M phosphate buffer (pH 7.2) 0.5 ml, 0.1 % methylene blue 0.5 ml and Na-lactate 200 μM. The total volume was 2.3 ml. Gas phase was air. The vessels were incubated at 37°C.

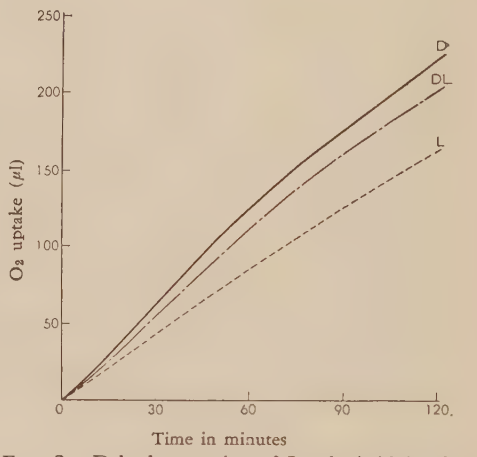


FIG. 2. Dehydrogenation of Lactic Acid by *Cl. acetobutylicum* grown in Lactate-added Speakman's Culture Medium.

Each Warburg vessel contained cell-suspension 1.0 ml (55 mg dried cells, racemiase activity 54.9 %), 0.2 M phosphate buffer (pH 7.2) 0.5 ml, 0.1 % methylene blue 0.5 ml and Na-lactate 200 μM. The total volume was 2.3 ml. Gas phase was air. The vessels were incubated at 37°C.

nearly equal extent. More distinct results were obtained from manometric assay of lactic dehydrogenase of racemiase-deficient cells grown in cyanide containing medium, as indicated in Fig. 3. The effect of cyanide on dehydrogenation of any optical isomer of lactic acids would be out of consideration, since very much the same

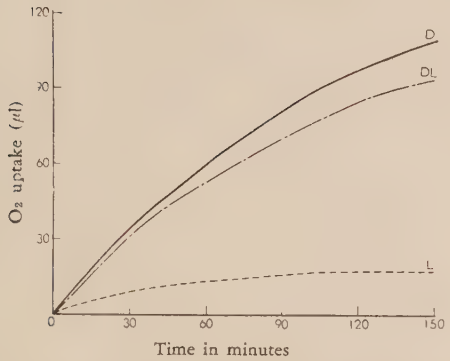


FIG. 3. Dehydrogenation of Lactic Acid by *Cl. acetobutylicum* grown in Cyanide-added Speakman's Culture Medium.

Each Warburg vessel contained cell-suspension 1.0 ml (74 mg dried cells, racemiase activity 6.4%), 0.2 M phosphate buffer (pH 7.0) 0.5 ml, 0.1% methylene blue 0.5ml and Na-lactate 90 μM. The total volume was 2.3 ml. Gas phase was air. The vessels were incubated at 37°C.

extent of inhibition of cyanide on dehydrogenation velocities was observed with each optical isomers of lactic acid as is shown in Table V.

TABLE V. INHIBITORY ACTION OF POTASSIUM CYANIDE ON THE DEHYDROGENATION OF LACTATE BY *Cl. acetobutylicum*

Each Warburg vessel contained glucose-grown cell-suspension of *Cl. acetobutylicum* 0.5 ml (dry matter 35 mg), 0.2 M phosphate buffer (pH 7.0) 0.5 ml, 0.1% methylene blue 0.5 ml and Na-lactate 0.5 ml (200 μM) to final volume 2.5 ml. Potassium cyanide was added to final concentration 0.01 M. The gas phase was air. Oxygen uptake was read for 130 minutes at 37°C.

Substrate	D(−)-Lactate	L(+)-Lactate	DL-Lactate
O_2 uptake for 130 min. (μl)			
without KCN	108	46	89
with KCN	32	11	26
Rate of inhibition (%)	70	76	71

Accordingly, it could be postulated that, *Cl. acetobutylicum* possesses D-lactic dehydrogenase in proper, and in racemiase-sufficient cells L-lactic acid would be dehydrogenated followed by conversion to D-form through the action of racemiase, which is affecting simultaneously in the reaction mixture. About this problem further discussion is presented below.

Oxidation of lactic acid by cell-suspension of *Cl. acetobutylicum*.

As described above, a new lactic acid forming system of redox potential higher than that of methylene blue was suggested. In this connection, the oxidation of both D- and L-lactic acids by cell-suspensions of *Cl. acetobutylicum* was investigated manometrically in the presence or absence of methylene blue. Figs. 4a and 4b as well as Figs. 5a and 5b indicate the results

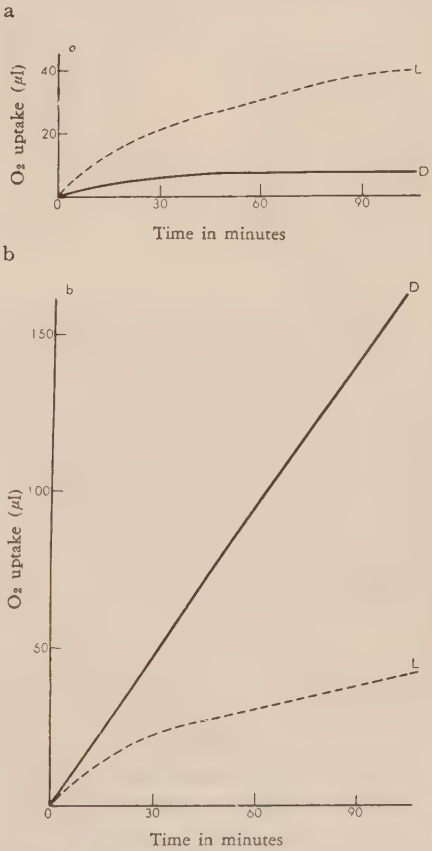


FIG. 4. Oxidation of Lactic Acid by *Cl. acetobutylicum* grown in Speakman's Gulture Medium (pH 7.0).

Each Warburg vessel contained cell-suspension 1.0 ml (43 mg dried cells), 0.2 M phosphate buffer (pH 7.0) 0.5 ml and Na-lactate 30 μM. The total volume was 2.3 ml. Gas phase was air. The vessels were incubated at 37°C.

a. Without methylene blue.

b. Supplemented with 0.5 ml of 0.1% methylene blue.

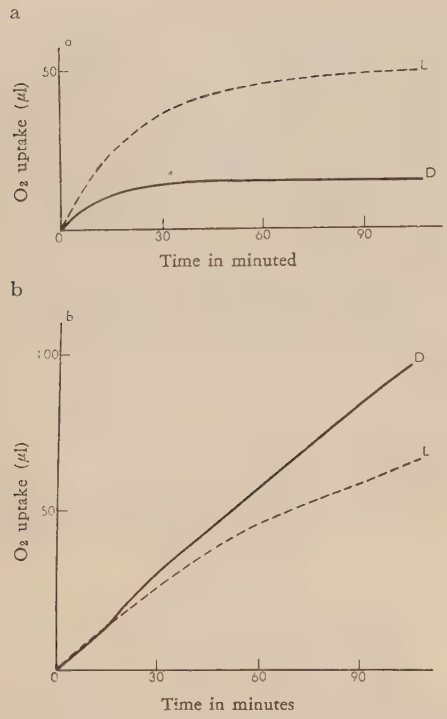


FIG. 5. Oxidation of Lactic Acid by *Cl. acetobutylicum* Grown in Speakman's Culture Medium (pH 5.2).

Each Warburg vessel contained cell-suspension 1.0 ml (43 mg dried cells), 0.2 M phosphate buffer (pH 5.2) 0.5 ml and Na-lactate 30 μM. The total volume was 2.3 ml. Gas phase was air. The vessels were incubated at 37°C.
a. Without methylene blue.
b. Supplemented with 0.5 ml of 0.1% methylene blue.

obtained from shaking experiments of cell-suspensions with D- or L-lactic acid in the presence or absence of methylene blue in Warburg's manometer at pH 7.0 and 5.2 respectively. Oxygen uptake with L-lactic acid was independent of the existence of methylene blue at each pH, and its velocity at pH 5.2 was somewhat higher than that at pH 7.0. On the contrary, methylene blue revealed intensive effect upon the oxygen uptake of D- lactic acid. In the absence of the dye, oxygen uptake of D-lactic acid was very few. At pH 7.0, oxygen uptake of D-lactic acid in the presence of the dye increased linearly in process of time far rapidly than at pH 5.2. In various experiments, it was pointed out that the maximum oxygen uptake of D-lactic acid in the presence of methylene blue was observed at pH 7.0 to 7.2, and the optimal pH range for the oxidation of L-lactic acid was below pH 6.0. It it presumable that the responsibility of the dye for the oxidation of D-lactic acid is generally due to reoxidation of leucodye formed in the conjugation with D-lactic dehydrogenase as follows:

$$\text{D-Lactate} \left(\begin{matrix}\nearrow \text{DPN} \\ \text{Pyruvate} \searrow \text{DPNH}\end{matrix}\right) \left(\begin{matrix}\nearrow \text{FH} \\ \searrow \text{F}\end{matrix}\right) \left(\begin{matrix}\nearrow \text{Mb} \\ \searrow \text{MbH}\end{matrix}\right) \left(\begin{matrix}\nearrow \text{H}_2\text{O}_2 \\ \searrow \text{O}_2\end{matrix}\right)$$

D-Lactic dehydrogenase — Diaphorase — (Air oxygen) (I)

$$\text{Pyruvic acid} + \text{H}_2\text{O}_2 \longrightarrow \text{Acetic acid} + \text{CO}_2 \quad \text{(Non-enzymic)} \quad \text{(II)}$$

$$\text{Pyruvic acid} + \tfrac{1}{2}\text{O}_2 \longrightarrow \text{Acetic acid} + \text{CO}_2 \quad \text{(Pyruvic oxidase)} \quad \text{(III)}$$

$$\text{H}_2\text{O}_2 \longrightarrow \text{H}_2\text{O} + \tfrac{1}{2}\text{O}_2 \quad \text{(Catalase)} \quad \text{(IV)}$$

and this organism is negative in catalase activity,

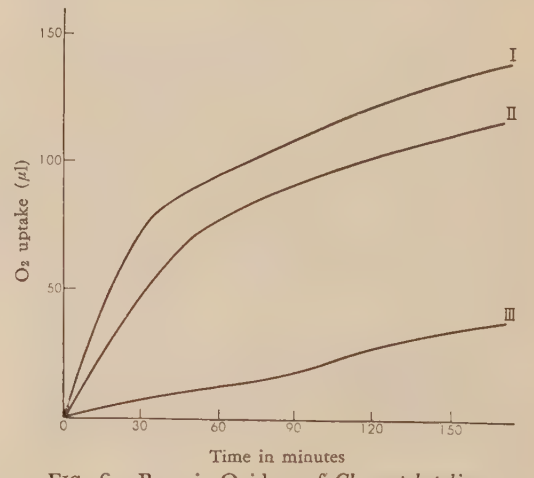


FIG. 6. Pyruvic Oxidase of *Cl. acetobutylicum*.

Each Warburg vessel contained cells obtained from Speakman's culture medium, 60 mg as dry matter, 100 μM Na-pyruvated and 0.5 ml of 0.2 M phosphate buffer (pH as indicated). The total volume was 2.3ml. Gas phase was air. The vessels were incubated at 37°C. Curves of CO_2 output were omitted from the figure.

Curve I. O_2 uptake at pH 5.2. R. Q. was 2.5.
Curve II. O_2 uptake at pH 6.4. R. Q. was 1.8.
Curve III. O_2 uptake at pH 7.2. R. Q. was 1.7.

and pyruvic oxidase of this organism shows merely few activity at pH 7 as indicated in Fig. 6, so that the reactions (I) and (II) would be actual in the experimental conditions described here.

On the whole, *Cl. acetobutylicum* seems to contain the two lactic acid metabolizing systems, one of them is independent to methylene blue and the other is corresponding to the dye. Baker[6] reported the existence of L-α-hydroxy acid oxidase in animal tissues, which was assumed to require flavin-mononucleotide as the cofactor and was distinguished from classical lactic dehydrogenase. *Mycobacterium phlei* was pointed out to possess L-lactic oxidase, which contained prosthetic flavin-adenine-dinucleotide and catalyzed direct oxidation of L-lactic acid to acetic acid[7~9]. Such lactic oxidases in other bacteria were further reported by Yamamura et al.[10] and Yagi et al.[11,12]. The foregoing fact that *Cl. acetobutylicum* oxidizes L-lactic acid without aid of any redox dyestuff would suggest the participation of such a lactic oxidase differing from classical lactic dehydrogenase. In this respect the responsibility of riboflavin, flavin-mononucleotide and flavin-adenine-dinucleotide for L-lactic acid oxidation by this organism was investigated, but no appreciable effect was observed. The isolation of L-lactic acid oxidizing system has been unsuccessful.

Distinctive action of *Cl. acetobutylicum* on D- and L-lactic acid.

Table VI indicates the summary of stoichiometric analysis in the metabolism of D- and L-lactic acid by glucose-lactate-grown cells of *Cl. acetobutylicum*. Following is the conclusion from the data in Table VI; i) ferrous ion slightly accelerated the disappearance of L-lactic acid, ii) L-lactic acid is consumed to a greater extent more than D-isomer, iii) remaining lactic acid contained both types of optical isomers, and the proportion of optical antipode to total remainder (e.g. the proportion of L-lactic acid to the total in the experiment employing D-lactic acid as the substrate) is raised by pre-existing ferrous ion, iv) significant amount of pyruvic acid can be delivered from D-lactic acid, but the accumulation of this acid from L-lactic acid is negligible despite of larger consumption of L-lactic acid more than D-isomer, and finally, v) both isomers of lactic acid are converted to equimolar acetic acid.

TABLE VI. METABOLISM OF D- AND L-LACTIC ACID BY GLUCOSE-LACTATE-GROWN CELLS OF *Cl. acetobutylicum*

Each incubation mixture contained intact cells of *Cl. acetobutylicum*, which was obtained from 0.15% DL-lactic acid added Speakman's culture medium, 1.13 g as dry matter, 0.1 M phosphate buffer (pH 5.2) 5 ml, $MgSO_4 \cdot 7H_2O$ 10 mg, $MnSO_4 \cdot H_2O$ 0.5 mg and NaCl 0.5 mg. The total volume was 50 ml. Gas phase was air. The mixture was incubated at 37°C for 72 hours.

Substrate	D(−)-Lactate		L(+)-Lactate	
Ferrous sulphate	omitted	20 mg%	omitted	20 mg%
Amount of acids (mM/50 ml)				
Initial lactic acid	6.00	6.00	5.11	5.11
Final lactic acid	5.32	5.50	3.11	2.66
D-Lactic acid	4.82	4.32	0.36	0.65
L-Lactic acid	0.50	1.18	2.75	2.01
Lactic acid consumed	0.68	0.50	2.00	2.45
Acetic acid	0.60	0.45	1.92	1.99
Pyruvic acid	0.016	0.007	trace	trace
Acetic formed/Lactic consumed	0.87	0.90	0.96	0.81

Responsibility of racemiase for lactic acid metabolism by *Cl. acetobutylicum*.

In the preceding articles it was pointed out that glucose-lactate-grown cells of *Cl. acetobutylicum* showed dehydrogenation efficiency on D- and L-lactic acid in the same extent, despite of the predominant dehydrogenation of D-lactic acid by glucose-grown cells. Apparent velocity of dehydrogenation of D-lactic acid was accelerated in parallel with the content of racemiase activity. On the contrary, the possession of particular L-lactic acid oxidizing enzyme in

6) C. G. Baker, *Arch. Biochem.*, **41**, 325 (1952).
7) N. L. Edson, *Biochem. J.*, **41**, 145 (1947).
8) W. B. Sutton, *J. Biol. Chem.*, **216**, 749 (1955).
9) F. B. Cousins, *Biochem. J.*, **64**, 297 (1956).
10) M. Kusunose, E. Kusunose and Y. Yamamura, *Kekkaku (Japan)*, **27**, 72, 243 (1952).
11) K. Yagi, S. Mitsuhashi and Y. Kojima, *Symposia on Enzyme Chemistry (Japan)*, **9**, 59 (1954).
12) S. Mitsuhashi, M. Murakami, K. Yagi and E. Suzuki, *Jap. J. Exp. Med.*, **22**, 1 (1952).

TABLE VII. INFLUENCE OF HYDROXYLAMINE ON THE DEHYDROGENATION OF LACTIC ACID

Cells of *Cl. acetobutylicum* harvested from DL-lactate-added culture and *Leuc. mesenteroides* from malt extract culture were used. Each Warburg vessel contained cell-suspension 1.0 ml (corresponding to 47 mg of *Cl. acetobutylicum* and 20 mg of *Leuc. mesenteroides* as dry matter), 0.2 M phosphate buffer (pH 7.2) 0.3 ml, 0.02 % methylene blue 0.5 ml and Na-lactate 0.3 ml (30 μM). The total volume was 2.3 ml. Gas phase was air. The vessels were incubated at 37°C for 150 minutes (*Cl. acetobutylicum*) and 90 minutes (*Leuc. mesenteroides*) respectively.

Organism	*Cl. acetobutylicum*				*Leuc. mesenteroides*	
Substrate	D(−)-Lactate		L(+)-Lactate		D(−)-Lactate	
Hydroxylamine*	none	added	none	added	none	added
O_2 uptake (μM)	10.8	12.6	10.3	2.3	6.8	3.0
Lactate consumed (μM)	9.7	12.5	9.2	2.2	5.5	4.7
Pyruvate found (μM)	1.0	2.9	1.0	0.3	1.2	2.2

* Per cent inhibition of racemiase by hydroxylamine otherwise examined was 73 and 95 with final concentration of 0.001 and 0.01M respectively.

this organism was assumed. To resolve this characteristic behaviour of this organism in relation with racemiase, a potent inhibitor of racemiase was employed in the dehydrogenation of lactic acid by glucose-lactate-grown cells. Cyanide and hydroxylamine inhibit racemiase most intensively among various inhibitors[13]. Here, hydroxylamine was used because it revealed no action on D-lactic dehydrogenase of *Cl. acetobutylicum* despite of the considerable extent of inhibitory action of cyanide on the dehydrogenase as indicated in Table V. Results were shown in Table VII. Dehydrogenation of L-lactic acid was markedly suppressed by the addition of hydroxylamine to final concentration 0.01M, though the dehydrogenation of D-lactic acid was not affected. Results with a D-lactic acid producing organism, *Leuconostoc mesenteroides*, were presented in the same table so as to compare with others.

From these results two cases would be suggested as follows. The first is that *Cl. acetobutylicum* possesses two types of lactic dehydrogenases, one of them is hydroxylamine sensitive L-lactic dehydrogenase and the other is non-susceptible D-lactic dehydrogenase. The former would somewhat resemble to D-lactic dehydrogenase of *Leuc. mesenteroides* in its behaviour towards hydroxylamine. The second case is that L-lactic acid is racemized followed by dehydrogenation by D-lactic dehydrogenase, and the suppression of dehydrogenation of L-lactic acid in the presence of hydroxylamine is due to inhibition of racemiase action preceding to D-lactic dehydrogenase action. In order to settle the problem, partial resolution of lactic dehydrogenase as well as racemiase was carried out. Cell-free racemiase was prepared from cultured filtrate of *Cl. acetobutylicum* by salting-out with ammonium sulphate (confer subsequent report). Extraction procedures of lactic acid dehydrogenase from this organism were described in Table VIII.

TABLE VIII. PREPARATION OF LACTIC DEHYDROGENASE FROM *Cl. acetobutylicum*

Glucose-lactate-grown dried cells 500 mg
↓ —ground with 1 g sea sand
↓ —extracted by 0.05M phosphate buffer (pH 7.0) 100 ml for 30 minutes.
Extract
↓ —adsorbed on tricalcium phosphate gel 5.4 g for 10 minutes
↓ —eluted with 0.2M phosphate buffer (pH 7.2) 100 ml overnight
Eluate
↓ —saturated with ammonium sulphate (0.6 saturation)
Precipitate
↓ —dissolved in 0.1M phosphate buffer (pH 7.2) 15 ml
↓ —added 0.6 volume of cold acetone (−15°C)
Precipitate
↓ —dissolved in water 5 ml
Crude prepartion of lactic dehydrogenase

As shown in Table IX, crude preparation of lactic dehydrogenase acted predominantly to D-lactic acid, and dehydrogenated L-lactic acid by

13) H. Katagiri et al., Subsequent report (unpublished).

TABLE IX. EFFECT OF RACEMIASE ON LACTIC DEHYDROGENASE OF *Cl. acetobutylicum*

Evacuated Thunberg-Borsook's tube contained crude preparation of lactic dehydrogenase (see Table VIII) 0.8 ml, boiled extract of *Cl. acetobutylicum* 0.3ml (corresponding to 100 mg dried cells), 0.2 M phosphate buffer (pH 7.2) 0.3 ml, 0.02% methylene blue 0.1 ml and Na-lactate 0.3 ml (30 μM). The total volume was 20 ml. Racemiase was prepared by salting-out from 100 ml filtrate of lactate-added culture and lyophilyzed. The tubes were incubated at 37°C.

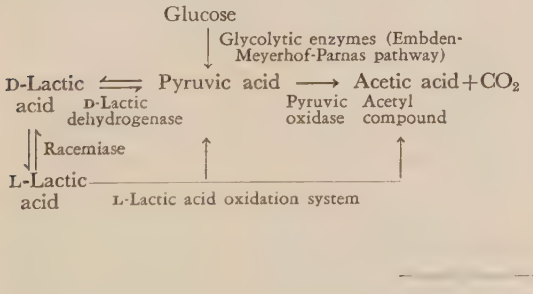
Substrate	Racemiase	Decoloration time (minutes)
None	—	>180
D(−)-Lactate	—	46
L(+)-Lactate	—	120
L(+)-Lactate	added	70
D(−)-Lactate	added*	>180
L(+)-Lactate	added*	>180

* Lactic dehydrogenase was omitted from the reaction mixture.

supplementing with racemiase though its velocity was still lower than that of D-lactic acid dehydrogenation. It was therefore assumed that this organism dehydrogenated L-lactic acid through the serial action of racemiase and D-lactic dehydrogenase.

It was also discussed in preceding articles that this organism was capable of oxidizing L-lactic acid by any specific enzyme system which was different from D-lactic dehydrogenase.

Summarizing the facts described above, the following metabolic sequence of both D- and L-lactic acid, considering the interaction of D-lactic dehydrogenase and L-lactic oxidizing system through racemiase, could be postulated.



In this scheme, it is still doubtful whether pyruvic acid would be an intermediate in the oxidation sequence of L-lactic acid to acetyl compound or acetic acid, though pyruvic acid could scarcely be detected in L-lactic acid metabolizing system as indicated in Table VI. Distinction of optimal pH supports the individuality of two types of lactic acid oxidizing (or dehydrogenating) systems. Moreover, it would emphasize the reliability of this scheme that the preference of oxidizing action on any one of the optical isomers of lactic acid was reversed by the addition of methylene blue, and that there could be observed no appreciable difference in dehydrogenation velocities between both optical isomers in the presence of sufficient amount of racemiase. Investigations on L-lactic acid oxidation system are now in succession.

SUMMARY

Behaviours of acetone-butanol fermentation bacterium, *Clostridium acetobutylicum*, towards optically active lactic acids were investigated. As a rule, cells harvested from lactic acid supplemented culture medium surpassed in metabolizing lactic acid as compared with glucose-grown cells. It was pointed out that *Cl. acetobutylicum* possessed D-lactic dehydrogenase by nature, and that L-lactic acid could be dehydrogenated after its conversion to D-isomer through the action of racemiase. In addition to D-lactic dehydrogenase, it was further indicated that this organism had another type of lactic dehydrogenase or oxidase, which was capable of oxidizing L-isomer with air oxygen in liberating acetic acid and carbon dioxide. Physiological action of racemiase as a mediator between two lactic enzymes, D-lactic dehydrogenase and D-lactic acid oxidizing enzyme, was ascertained.

In addition, it is mentioned about pyruvic oxidase and partial inhibition by cyanide to lactic dehydrogenase.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2, p. 182~187, 1960]

Microbiological Studies of *Coli-aerogenes* Bacteria*

Part X. Various Factors Influencing α-Ketoglutarate-Fermentation

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α-Ketoglutarate was formed from the various carbohydrates including lactose, maltose, sucrose, D-glucose, D-fructose, D-galactose, D-mannose, D-mannitol, L-rhamnose, D-xylose, L-arabinose and glycerin. The influence of pH of the reaction mixture were tested, and inorganic phosphate was observed to be indispensable for α-ketoglutarate-fermentation. A cell of *E. coli* grown statically on glucose was found to reveal an ability of producing α-ketoglutarate under aerobic conditions. Optically dextro lactic acid was potent in the formation of α-ketoglutaric acid. The following reagents revealed the inhibiting effect on α-ketoglutarate-fermentation; $CuSO_4$, $AgNO_3$, iodoacetate, 2, 4-dinitrophenol, NaN_3, 3-sulfanilamido-6-methoxypyridazine and arsenite, while, kanamycin and 8-azaguanine has no inhibiting effect. When *E. coli* was grown in a glucose-medium, a small supply of air increased the yield of acetate against decreasing α-ketoglutarate.

INTRODUCTION

In the previous papers of this series, the authors have studied the oxidative fermentation of the varied carbonaceus substances including glucose, acetic, pyruvic, D** or DL-lactic and C_4-dicarboxylic acids by the bacteria of *coli-aerogenes*, and have found the fact that these bacteria induce α-ketoglutaric acid-fermentation under shaking conditions[1~5]. Further investigations have been undertaken with the object of throwing some light on the mechanism of the metabolisms of C_6-tricarboxylic acids in *coli-aerogenes*[5,7~10]. The authors have also investigated some environmental factors influencing the metabolism of substrate-carbon, and it has been demonstrated that the mode of the oxidative fermentation in the bacteria is greatly affected by the chemical factors such as: the content of nitrogen-sources[2] or vitamin B_1 in the growth-medium[2]; the presence or absence of inorganic iron[4], arsenic compounds[3] and of various antibiotics including dihydrostreptomycin, chloramphenicol and tetracyclines in either growth-medium or in the reaction-mixtures containing non-proliferating cells of the bacteria[6]. The present paper again deals with the metabolic variation due to chemical and physical factors.

EXPERIMENTAL AND RESULTS

Methods. Experimental procedures were performed by the methods mentioned in the previous papers[1~10]. Kanamycin and lederkyn (3-sulfanilamido-6-methoxypyridazine) were obtained from the Meiji Seika Kaisha, Ltd. (Tokyo, Japan) and the Takeda Pharmaceutical Industries, Ltd. (Osaka, Japan), respectively. Optically L-lactic acid was generously supplied by Mr. T. Sugimori of this laboratory.

* A part of this paper was read before the Meeting of the Agricultural Chemical Society of Japan (April, 1955).

** In the previous papers (Parts IV and V), the symbol of the optically active lactic acid must be corrected: L as D, according to misuse of the capital letter.

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2) H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 215 (1957).
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10) H. Katagiri and T. Tochikura, This Bulletin, **23**, 489 (1959).

TABLE I. PRODUCTION OF α-KETOGLUTARIC ACID FROM VARIOUS CARBOHYDRATES BY *E. Coli*

Carbon-sources	(%)	Time of incubation (days)	α-Ketoglutaric acid found (g/100 ml)	Pyruvic acid found
Maltose	5.0	5	0.98	−
Lactose	5.0	5	0.99	+
Sucrose	5.0	4	0.49	+
Koji ext.	4.8 (as glucose)	4	1.25	−
D-Glucose	5.0	4	2.29	−
D-Fructose	5.0	4	2.15	−
D-Mannose	5.0	4	1.55	−
D-Mannitol	4.0	4	0.58	−
D-Galactose	4.0	4	0.54	+
L-Rhamnose	5.0	5	0.88	−
D-Xylose	4.0	4	0.48	++
L-Arabinose	4.0	4	0.65	++
Glycerin	3.0	4	0.57	−

Production of α-Ketoglutarate from Various Carbohydrates by *E. coli*. In the previous papers[1~6], the authors investigated the production of α-ketoglutaric acid by the bacteria of *coli-aerogenes* with varied sources of carbon including glucose, acetic, pyruvic, lactic, C_4-dicarboxylic and C_6-tricarboxylic acids. Among various sources of carbon, good yields of α-ketoglutaric acid were obtained with glucose, pyruvic, lactic and C_4-dicarboxylic acids, whereas, the exceedingly low yields were ascertained with acetic and C_6-tricarboxylic acids. In the present paper, experiments of the growing culture of *E. coli* were again instituted with various carbohydrates. *E. coli* (G-2 strain) was grown in the media containing 3~5% various carbohydrates, 0.05~0.10% $(NH_4)_2HPO_4$, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% NaCl and 0.05% $MgSO_4 \cdot 7H_2O$ in tap water, to which sterilized $CaCO_3$, corresponding to 75% of carbon-sources was added at the time of inoculation. The medium (100 ml) was taken in a 500 ml-flask and sterilized. After inoculation with 0.5 ml of 40 hours' culture at 37°C under static conditions of the organism on bouillon, the flasks were placed on a shaker at 30°C for 4~5 days. The results of experiments are shown in Table I, from which it will be seen that α-ketoglutarate was produced with all carbohydrates tested; maltose, lactose, sucrose, koji ext. D-glucose, D-fructose, D-mannose, D-mannitol, D-galactose, L-rhamnose, D-xylose, L-arabinose and glycerin, but remarkable difference was pointed out among the yields of α-ketoglutarate, high yields being obtained with glucose, fructose and mannose,

Influence of pH of Medium upon the Production of α-Ketoglutarate. The effect of pH of medium upon α-ketoglutarate-fermentation by washed cells of *E. coli* is shown in Table II: incubations were carried out on a shaker at 30°C for 5~12 hours on the media (35~50 ml) containing 10 mM phosphate buffer, 60~100 μM $MgSO_4$, stated amounts of Na-salts of organic acids and 120~140 mg washed cells of *E. coli* (G-2 strain) grown aerobically for 1~2 days on a glucose-ammonium-medium as mentioned in the previous papers[3~6]. As

TABLE II. INFLUENCE OF pH OF MEDIA UPON PRODUCTION OF α-KETOGLUTARATE BY *E. coli*

(a) With 2.0 mM Na-pyruvate and 140 mg bacterial cells; total volume 35 ml; 5 hours' incubation.

pH	5.5	5.8	8.2
Pyruvate consumed (mM)	2.000	2.000	2.000
α-Ketoglutarate found (mM)	0.652	0.740	0.397

(b) With 3.6 mM Na-pyruvate, 2.0 mM Na-succinate and 120 mg bacterial cells; total volume 50 ml; 12 hours' incubation.

Substrates	Pyruvate		Succinate	
pH	5.3	7.6	5.3	7.6
α-Ketoglutarate found	0.775	0.700	0.390	0.273

(c) With 4.0 mM Na-acetate and 120 mg bacterial cells; total volume 50 ml; 12 hours' incubation.

Exp. No.	I–a	I–b	II–a	II–b
pH	5.3	7.6	5.2	7.7
α-Ketoglutarate found (mM)	Trace	0.09	Trace	0.15

it will be seen in Table II, the higher yields of α-ketoglutaric acid were obtained in acidic media than in alkaline media when the washed cells of *E. coli* were incubated in the presence of C_3- or C_4-acids, while the yield was increased under alkaline conditions when acetate was used as a sole source of carbon.

Production of α-Ketoglutarate by a Cell of *E. coli* grown under Anaerobic (Static) Conditions. In the investigations on α-ketoglutarate-fermentation hitherto mentioned, the experiments were carried out with the growing cultures under aerobic conditions or with the washed and treated cells and cell-extracts of the bacteria obtained by aerobic cultivation. In the present experiments, *E. coli* was grown under anaerobic (static) conditions in order to ascertain whether the anaerobically grown-cell would possess an ability to induce α-ketoglutarate-fermentation.

The oxidative fermentation by statically grown-cells

was at first carried out in the following manner. The medium employed here contained 2.0% glucose, 0.08% $(NH_4)_2HPO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% NaCl and 1% $CaCO_3$. One hundred ml of the medium was dispensed in a 500 ml-shaking flask. After inoculation with *E. coli* (G-2 strain), the medium was incubated at 37°C for 120 hours under static conditions, and thereafter the shaking flask was placed on a shaker and incubation was continued at 30°C for 30 hours under aerobic conditions. Another flask without any shaking procedure was taken for comparison. The results

Table III. Oxidative Fermentation by Statically Grown-cells of *E. coli*

	Static culture		Shaking culture for 30 hours after 120 hours' static incubation
	For 120 h.	For 150 h.	
Glucose consumed (%)	100.0	100.0	100.0
Lactic acid found (mg/dl)	835.5	810.0	150.8
α-Ketoglutaric acid found (mg/dl)	0	0	230.5

of experiments are shown in Table III. It was observed that the fermentation of glucose by *E. coli* gave rise to a large amount of lactate under static conditions, but when the bacterial broth, in which the anaerobic fermentation of glucose had proceeded to completion, was exposed to shaking conditions, the remarkable production of α-ketoglutarate occurred against the disappearance of lactate. Subsequently, another experiment was made with a statically grown-cell of *E. coli*. The organism was grown under static conditions at 37°C for 4 days in a medium containing 2% glucose, 0.1% $(NH_4)_2HPO_4$, 0.15% $(NH_4)_2SO^4$ and other mineral matters as already mentioned. After incubation, cells were collected by a centrifuge and washed twice with distilled water. Reaction mixture (40 ml) contained 10 mM phosphate buffer (pH: 5.7), 50 μM $MgSO_4$, 3.33 mM DL-Na-lactate and 250 mg washed cells of *E. coli*. The yield of α-ketoglutarate was observed to be 0.67 mM per 40 ml after 8 hours' incubation under aerobic conditions. Thus, a statically grown-cell of *E. coli* has also been found to reveal a potency of producing α-ketoglutarate from lactate.

Production of α-Ketoglutaric Acid from L-Lactic Acid. The bacteria of *coli-aerogenes* are well known to produce D-lactic acid from glucose. It is moreover demonstrated by Katagiri and Kitahara[11,12] that both *E. coli* and *B. lactis aerogenes* can not produce racemization of L-lactic acid, i.e., the cells of these organisms are devoid of an enzyme termed racemiase (lactic acid racemase), although some strains of bacteria including so-called lactic acid-bacteria, *Staphylococcus ureae* and *Clostridium acetobutylicum* are able to produce the enzyme under suitable conditions. In the previous of this series[4], the authors have found that the oxidations of D- and DL-lactic acids by *coli-aerogenes* actually give rise to a large amount of α-ketoglutaric acid. In the present paper, experiments were carried out with L-lactic acid; reaction mixtures (40 ml) contained 10 mM phosphate buffer (pH: 6.4) 60 μM $MgSO_4$, the requisite amounts of Na-salts of L-lactic and acetic acids and 140 mg washed cells of *E. coli* (G-2) grown aerobically for 2 days at 30°C on 2% glucose-ammonium-medium in the same manner as already mentioned, and the mixtures were kept on a shaker at 30°C for 10 hours. The results are shown in Table IV. It will be seen that a cell of

Table IV. Production of α-Ketoglutaric Acid from L-Lactic Acid by Washed Cells of *E. Coli*

L-Lactic acid added (mM)	3.330	3.330
Acetic acid added (mM)	0	2.000
10 hours' incubation		
L-Lactic acid consumed (mM)	2.730	2.330
α-Ketoglutaric acid found (mM)	0.413	0.713
(Molar yield)	0.151	0.305

E. coli grown aerobically on glucose possesses an ability to produce α-ketogluaric acid from L-lactic acid which is not produced by the fermentation of glucose by the organism, and that the addition of acetic acid to a L-lactic acid-containing medium also brings about an increase in the yield of this C_5-keto-acid.

Effects of Various Reagents on α-Ketoglutarate-Fermentation. Table V shows the influence of various reagents on the formation of α-ketoglutarate by the washed cells of *E. coli* (G-2 strain) grown aerobically on glucose. Incubations were carried out at 30°C on a shaker with the media (15~40 ml) containing 1.5~5.0 mM phosphate buffer, 40~100 μM $MgSO_4$, the stated amounts of substrates (Na-salts), various reagents and of washed cells. The results may be summarized as follows. Reagents such as iodoacetate, 2.4-dinitrophenol, $CuSO_4$, $AgNO_3$ and arsenite strongly inhibited the degradation of substrates. Sodium-azide also revealed the inhibiting effect. Kanamycin and 8-azaguanine (8-AG) had no inhibiting effect on α-ketoglutarate-

11) H. Katagiri and K. Kitahara, *J. Agr. Chem. Soc., Japan*, **11**, 997 (1935); **12**, 96 (1936); **12**, 281 (1936); **12**, 844 (1936); **12**, 1217 (1936).

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TABLE V. INFLUENCE OF VARIOUS REAGENTS UPON PRODUCTION OF α-KETOGLUTARATE BY *E. Coli*

Exp. No.	Time of incubation (hours)	Washed cells (mg)	Substrate added (mM) Pyruvate		Inhibitors added	Total volume (ml)	pH	Pyruvate found (mM)	α-K.G. found (mM)
					$CuSO_4$ (M/l)				
I–a	12	86	2.000		5.3×10^{-3}	15	6.4	1.340	Trace
I–b	12	86	2.000		0	15	6.4	0	0.474
					$AgNO_3$ (M/l)				
II–a	10	110	4.000		1.0×10^{-3}	40	6.0	4.000	0
II–b	10	110	4.000		0	40	6.0	0	1.200
					Iodoacetate (M/l)				
III–a	12	70	2.000		2×10^{-3}	25	6.0	1.485	0
III–b	12	70	2.000		0	25	6.0	0	0.493
					Arsenite (M/l)				
III–c	12	70	2.000		1×10^{-2}	25	6.0	1.930	0
					2, 4-Dinitrophenol				
IV–a	5	140	2.000		3.7×10^{-4}	35	5.5	1.775	0
IV–b	5	140	2.000		0	35	5.5	0	0.600
			Pyruvate+acetate		Lderkyn (γ/ml)				
V–a	11	100	3.000	2.000	1700	36	5.7	0	0.235
V–b	11	100	3.000	2.000	0	36	5.7	0	0.713
V–c	11	100	3.000	2.000	1700	36	7.5	0	0.357
V–d	11	100	3.000	2.000	0	36	7.5	0	0.710
					8−A.G (γ/ml)				
V–e	11	100	3.000	2.000	490	36	7.5	0	0.803
V–f	11	100	3.000	2.000	980	36	7.5	0	0.850
					Kanamycin sulfate (γ/ml)				
VI-a	12	85	2.000	2.000	860	35	6.0	0	0.755
VI–b	12	85	2.000	2.000	0	35	6.0	0	0.808
			Glucose ($CaCO_3$ added)						
VII–a	12	85	2.000 (2.0)		806	35	6.0	0	0.432
VII–b	12	85	2.000 (2.0)		0	35	6.0	0	0.472
			D-Lactate+acetate		NaN_3 (M/l)				
VIII–a	12	180	2.500	2.000	0	50	6.3		0.585
VIII–b	12	180	2.500	2.000	5×10^{-3}	50	6.3		0.125

TABLE VI. EFFECT OF LEDERKYN ON OXIDATION OF α-KETOGLUTARATE BY *E. coli*

Lederkyn added (γ/ml)	0	570	1140
α-Ketoglutarate consumed (%)	66	70	70

Reaction mixture contained 5 mM phosphate buffer (pH: 6.0), 100 μM $MgSO_4$, 500 μM Na-α-ketoglutarate, 100 mg washed cells of *E. coli* (G-2-strain) grown aerobically on glucose, and stated amounts of lederkyn; total volume 35 ml; 10 hours' incubation at 30°C on a shaker.

TABLE VII. EFFECT OF INORGANIC PHOSPHATE ON PRODUCTION OF α-KETOGLUTARATE FROM PYRUVATE BY *E. coli*

Kind of buffer (mM/50 ml)	Citrate-HCl buffer 5.0		Borate-HCl buffer 3.0	
Phosphate added (μM/ml)	0	20	0	20
Incubation for 12 hours on a shaker.				
Pyruvate consumed (mM)	3.300	4.000	3.500	4.000
α-Ketoglutarate found (mM)	0.205	0.740	0.136	0.350

fermentation. The presence of lederkyn (3-sulfanil amido-6-methoxy-pyridazine) brought about the remarkable decrease in the yield of α-ketoglutatate without causing any inhibition of the oxidative removal of substrate. In the previous paper[6], the authors have reported the stimulating effect of dihydrostreptomycin on the oxidation of α-ketoglutarate by *E. coli*. From the results shown in Table VI, however, it appears that lederkyn has no stimulating effect on the oxidation of α-ketoglutarate by *E. coli*.

The results of experiments given in Table VII shows the effect of inorganic phosphate upon the production

TABLE VIII. OXIDATION OF GLUCOSE BY *E. coli* UNDER VARIOUS CONDITIONS

Method of aeration	Bubbling a stream of air with suction pump		Bubbling a stream of air with compressed air cylinder			Aeration with a shaker	
Volume of media	150 ml in 300 ml Erlenmeyer flask		1000 ml in 2000 ml cylinder			50~100 ml in 500 ml shaking flask	
Exp. No.	I	II	III	IV	V	VI	VII
Initial conc. of glucose (%)	2.0	2.0	2.0	2.0	5.0	2.0	5.0
Conc. of $(NH_4)_2HPO_4$ (%)	0.05	0.05	0.05	0.10	0.10	0.05	0.10
Conc. of $(NH_4)_2SO_4$ (%)	0	0	0	0	0.10	0	0.10
Temperature of incubation (°C)	37	37	30	30	30	30	30
Time of fermentation (days)	3	4	4	2	4	5	4
Consumption of glucose (%)	100	100	100	100	100	100	100
Products found after incubation (% on consumed glucose)							
Acetate	25.0	24.0	25.5	32.8	30.4	0	0
Pyruvate	Trace	0.5	4.5	1.2	Trace	0	0
α-Ketoglutarate	2.9	3.5	3.6	3.7	5.3	42.3	46.3

of α-ketoglutarate from pyruvate. The washed cells (150 mg) of *E. coli* (G-2 strain) aerobically grown on glucose were incubated on a shaker at 30°C with 50 ml of the media containing indicated buffers (pH 6.5), 100 μM $MgSO_4$ and 4.0 mM Na-pyruvate in the presence and absence of Na_2HPO_4-KH_2PO_4. It has been observed that inorganic phosphate is indispensable for the α-ketoglutarate-producing reaction with pyruvate. The presence of borate also resulted in the low yield of α-ketoglutarate.

Influence of Air-supply upon the Oxidative Fermentation of Glucose by *E. coli*. The mode of metabolism of glucose in the bacteria of *coli-aerogenes* has already been known to depend on the amount of available oxygen present in the media. Anaerobically, the synthesis of the bacterial cells occurs at their minimum levels and most of glucose-carbon is converted into lactase, formate, acetate, succinate, pyruvate, ethanol, acetoin, 2:3-butanediol, hydrogen gas and carbon dioxide[13~16]. Aerobically, most of glucose-carbon is converted into α-ketoglutarate and carbon dioxide, being accompanied with the good yield of the bacterial cells, although some strains of the organisms produce acetate, pyruvate and acetoine in addition to α-ketoglutarate and carbon dioxide[1~3]. In the present paper, the authors' interest has been directed to the influence of air-supply upon the metabolism of glucose in *E. coli*. It was grown aerobically by bubbling a gentle stream of sterile air into a cultural medium; air was supplied to the cultural medium by a compressed air cylinder or by suction pump and was passed through a close-packed cotton filter to ensure sterility. The medium containd 2~5% glucose, 0.05~0.1% $(NH_4)_2HPO_4$, the requisite amounts of $(NH_4)_2SO_4$ and other mineral salts as already mentioned. Experiments with a shaker were also carried out for the sake of comparison. According to the method of Cooper et al[17], a much greater amount of oxygen was ascertained to be supplied by aeration with a shaker than by bubbling a stream of air. The results of experiments are shown in Table VIII. It was found that when *E. coli* was aerobically grown by bubbling a gentle stream of air into the media, a remarkable production of acetate occurred, the yield being about 24~33% on consumed glucose, but α-ketoglutarate was produced only in a very small amount. It should, moreover, be noted that aeration by a gentle stream of air is accompanied with the formation of pyruvate during the early stage of fermentation, although this keto-acid is oxidized to acetate at the last period of incubation. On the other hand, the yield of α-ketoglutaric acid obtained under shaking conditions may be given according to the following equation, based on the experimental results mentioned in the previous papers[1~3].

$$3C_6H_{12}O_6 + 10O_2 \longrightarrow 2C_5H_6O_5 + 8CO_2 + 12H_2O$$

Molar yield; about 0.667

(% on consumed glucose; about 54)

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SUMMARY

1) *E. coli* was able to produce α-ketoglutarate when the organism was grown aerobically on the media containing various kinds of carbohydrates; carbohydrates tested were lactose, maltose, sucrose, D-glucose, D-fructose, D-galactose, D-mannose, D-mannitol, L-rhamnose, D-xylose, L-arabinose and glycerin.

2) The effects of the pH of the media upon α-ketoglutarate-fermentation were investigated.

3) A striking effect of inorganic phosphate was observed on the production of α-ketoglutarate.

4) A statically grown cell of *E. coli* was found to be capable of inducing α-ketoglutarate-fermentation.

5) L lactic acid was potent in the production of α-ketoglutarate.

6) The action of various reagents was investigated and the inhibiting effects on α-ketoglutarate-fermentation were observed with $CuSO_4$, $AgNO_3$, iodoacetate, 2,4-dinitrophenol, NaN_3, 3-sulfanil-amido-6-methoxy pyridazine and arsenite.

7) A small supply of air into a glucose-medium increased the yield of acetate against decreasing α-ketoglutarate.

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Microbiological Studies of *Coli-aerogenes* Bacteria*

Part XI. Influence of the Amount of Available Oxygen upon α-Ketoglutarate-fermentation

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Investigations were carried out on the effect of the amount of available oxygen upon the metabolisms of carbonaceous substances in *coli-aerogenes* bacteria. When *E. coli* was grown in the presence of glucose, a gentle aeration (a small supply of air) suppressed the accumulation of lactate against increasing the productions of acetate, carbon dioxide and of the bacterial cell. α-Ketoglutarate-fermentation occurred only under a large supply of air. When the cells of *coli-aerogenes* were incubated under the low levels of oxygen, not only glucose, C_3- (pyruvic, lactic), C_4-dicarboxylic and C_6-tricarboxylic acids but also acetic acid were mainly oxidized to carbon dioxide and water, but little or no formation of α-ketoglutaric acid occurred. The production of α-ketoglutaric acid from various organic acids except C_6-tricarboxylic acids also took place only under the high levels of available oxygen. It was concluded that the direction of oxidative metabolism of substrate-carbon was changeable according to variation of the environmental oxygen level. Relationships among respiration, oxidative fermentation and oxidative assimilation were investigated under various levels of available oxygen.

INTRODUCTION

In the previous papers of this series, the authors have found that lactic acid-fermentation, which occurs anaerobically, by a number of *coli-aerogenes* bacteria is converted into α-ketoglutaric acid-fermentation under aerobic conditions[1~3]. The oxidative metabolisms in the bacteria have, furthermore, been studied with varied substrates including carbohydrates, C_2–, C_3–, C_4- and C_6- acids, and consequently the mode of degradations of C_6-tricalboxyric acids was propounded and discussed[4~11].

The effects of oxygen-supply on the fate of substrate-carbon in the metabolism of bacteria have been studied by several workers[13~17]. In the preceding paper[12], the authors have also demonstrated that when *Escherichia coli* is aerobically cultivated with a gentle stream of sterile air in the presence of glucose, ammonium-nitrogen and other mineral salts, a large amount of acetic acid is obtained in place of lactic and α-ketoglutaric acids as the fermentation-product[12].

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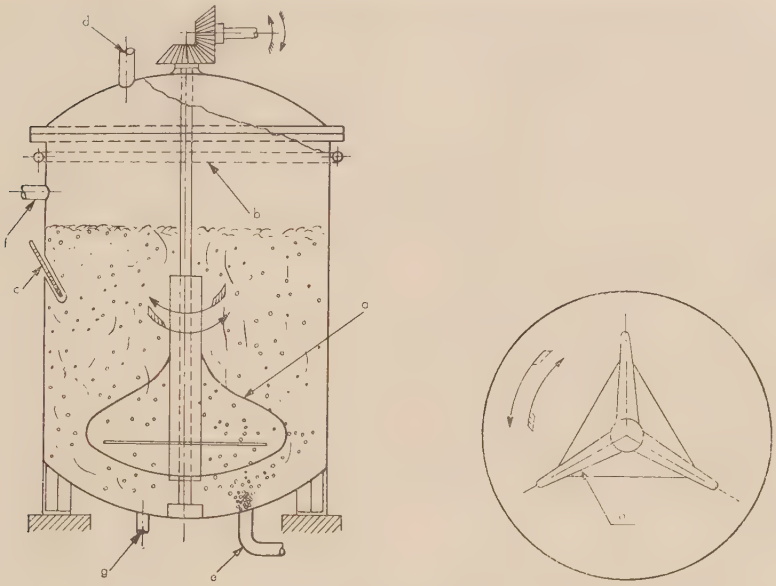

FIG. 1. Diagram of 10-liter Swinging Tank (K-Type Fermentor)

a. Periodic Agitator (Clock and anti-clockwise motion with angle $\pi/6$ to π)
b. Cooling water Pipe, c. Thermometer, d. Air Outlet,
e. Air Inlet, f. Raw Material Inlet, g. Fermented Liquor Outlet

The influence of the amount of available oxygen upon the fate of substrate-carbon in *coli-aerogenes* are again discribed in the present paper.

EXPERIMENTAL AND RESULTS

Methods. Many experiments described in this paper were carried out with *Escherichia coli* (G-2 strain). *Aerobacter aerogenes* (B-2 strain) was also used in some experiments. Analytical procedures were performed by the methods mentioned in the previous papers[1,6].

Fermentations were run in 10-liter stainless steel tank (Fig. 1) equipped with propellers. This fermentor has been termed a swinging (K-type) tank by the authors[23]. Air was passed through a sterile cotton filter and introduced into a cultural medium through a sparger beneath an agitator with three propellers. Fermentation-temperature was controlled at 30°C by circulating water through the jacket. Some experiments were also carried out with a shaker or the conventional Warburg-respirator as already mentioned in the previous papers.

Aeration Efficiency. The transfer of oxygen from the gas phase to the liquid phase is generally recognized to be the limiting factor in aeration of cultures. The aeration efficiency is referred to in this paper as sulfite oxidation value, expressed as mM O_2 per liter per min. Effective aeration values were determined by the oxidation of sulfite in the presence of copper according to the method of Cooper et al.[16]. Effective aeration rates of some aeration devices employed in the authors' laboratory are shown in Table I. In the case of the fermentation-tanks with 5~10 liters of medium, it will be seen that aeration without any agitation gives very poor aeration efficiencies, and in order to obtain high aeration efficiencies, aeration must be accompanied with agitation of the medium. Shake flasks with 15~100 ml of a medium on a shaker were observed to be effective (maximal sulfite value; about 2.9 mM O_2 per liter per min.), whereas the conventional Warburg-apparatus revealed poor aeration efficiencies (0.15~0.35 mM O_2 per liter per min.).

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TABLE I. EFFECTIVE AERATION RATES OF SOME AERATION PROCEDURES

Vessel used	Volume of medium (ml)	Aeration procedure	Aerating rate of air (vol per vol per min.)	Effective aeration (mM O_2 per L per min.)
(a) Conventional Warburg vessel	2.5~5.0	Shaken on Warburg respirator	——	0.15~0.35
(b) 500 ml shaking flask	15~100	Shaken on shaker at 110~130 r. p. m.	——	0.93~2.92
(c) Conventional submerged fermentor	5000~7000	120 r. p. m. agitator plus sparger	0.50	0.02
			1.00	0.03
(d) K-type fermentor (swinging fermentor)	7000	300 r. p. m. agitator plus sparger	1.00	0.28
	8000~10000	Aeration only with sparger	1.00	0.08~0.11
		63 r. p. m. agitator plus sparger	0.50	0.25
			1.00	0.40
			0.25	0.12
			0.13	0.09
		100 r. p. m. agitator plus sparger	0.20	0.47~0.52
		110 r. p. m. agitator plus sparger	0.55	0.91
		132 r. p. m. agitator plus sparger	0.45	1.46
		138 r. p. m. agitator plus sparger	0.55	1.62
			1.00	2.20
		168 r. p. m. agitator plus sparger	1.00	3.00

TABLE II. OXIDATIVE FERMENTATION BY *E. coli* UNDER LOW AND HIGH LEVELS OF AVAILABLE OXYGEN

Time of fermentation (hours) at 30°C	23	38	48	64
Methods of aerobic cultures				
Aerating rate of air (vol per vol per min.)	1.0	1.0	1.0	1.0
Agitation	—	— After 38 hours' incubation agitator (120 r. p. m.) was operated		
Consumption of glucose (mM/dl)	15.10	18.40	18.40	18.40
(%)	82.2	100.0	100.0	100.0
Acetic acid found (mM/dl)	8.10	11.00	—	8.00
(Molar yield)	0.536	0.597		0.435
Lactic acid found (mM/dl)	10.70	13.50	10.50	7.400
(Molar yield)	0.710	0.734	0.571	0.402
Pyruvic acid found (mM/dl)	1.50	0	0	0
(Molary yield)	0.099			
α-Ketoglutaric acid found (mM/dl)	Trace	0	0.51	1.55
(Molar yield)			0.028	0.084
Bacterial cells found (mg/dl)	192	272	296	326

Note: Fermentation without any aeration and agitation was as follows:

Time of incubation (hours)	Glucose consumed (mM/dl)	Acetic acid found (Molar yield)	Lactic acid found (Molar yield)	Bacterial cells found (mg/dl)
64	10.8	0.485	0.950	35

Oxidative Fermentation in Fermentor. Table II shows the effects of aeration with a stream of sterile air and agitation upon the fermentations of glucose in *E. coli* (G-2 strain). The medium contained 3.31% (18.4 mM/dl) glucose, 0.1% each $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% NaCl and 5 p.p.m. $Fe_2(SO_4)_3 \cdot 7H_2O$ in tap water to which sterilized $CaCO_3$ corresponding to 75% of glucose was added at the time of inoculation. Ten liter of the medium was taken in a fermentor and sterilized. After inoculation with 200 ml of 60 hours' culture under aerobic conditions at 30°C of the organism on glucose-medium, the incubation was carried out at 30°C under the conditions as indicated in Table II. During the first phase of fermentation, the medium was aerated only by a bubbling stream of air, without any agitation by propellers; this period of fermentation was about 38 hours with a medium containing 3.31% glucose. In the second phase of fermentation, i.e., when glucose was completely consumed, an agitator was operated in the fermentor to increase the efficiency of aeration. An experiment of anaerobic culture was made with the same medium for the sake of comparison. The chemical changes occurring during fermentation is shown in Table II. Lactate was observed to be produced with glucose even under such conditions that the medium was aerated with a stream of air but without any agitation, while the yield of the bacterial cell was increased under the conditions. It has, however, been found that the formation of α-ketoglutarate occurs when the fermented liquid is again incubated by the combination of aeration with a stream of air and agitation by propeller.

Similar experiments were carried out with a medium containing 2.97% (16.5 mM) glucose, 0.42% Na-glutamate·H_2O, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 500 γ% $FeSO_4 \cdot 7H_2O$, 0.2% NaCl and 2% $CaCO_3$. In the first stage of fermentation, the medium was acreated at 30°C by a stream of air at rate of volume per volume per minute, without any agitation, and this time of incubation was about 70 hours. In the second stage (after 70 hours' incubation), the agitator was operated to increase the efficiency of aeration. The results of experiment are shown in Table III, from which the following facts will be indicated. The consumption of glucose was observed to proceed at a much faster rate in aerated culture than in anaerobic culture without any aeration. In the case of aerated culture with a stream of air, the molar yields of acetate and lactate after 38 hours' incubation, were about 0.91 and 0.67, respectively, and also those after 60 hours were about 1.21 and 0.42. In these cases, however, α-ketoglutarate was never formed in a noticeable amount. On the other hand, the molar yields of acetate and lactate under anaerobic conditions were about 0.71 and 0.84, respectively after 87 hours' incubation. The yield of the bacterial cell synthesized was ascertained to be exceedingly high even in a gentle aeration as shown in Table III. Thus, it is clear that when a small amount of oxygen is supplied to the medium, α-ketoglutarate-fermentation does not occur, but there are the increasing yields of acetate and bacterial cells against the decreasing yield of lactate. It should be noted, here, that *coli-aerogenes* bacteria are capable of inducing α-ketoglutarate-fermentation only under a high level of available oxygen

TABLE III. FERMENTATION OF GLUCOSE BY *E. coli* UNDER ANAEROBIC AND AEROBIC CONDITIONS

Time of fermentation at 30°C (hours)		38		60		87	
Kinds of cultures		Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic
Aerating rate of air (vol per vol per min.)		—	1.0	—	1.0	—	1.0
Agitation		—	—	—	—	After 70 hours, agitator (140r.p.m.) was operated	
Consumption of glucose	(mM/dl)	6.30	16.50	10.10	16.50	13.30	16.50
	(%)	38.2	100.0	61.3	100.0	80.7	100.0
Acetic acid found	(mM/dl)	—	15.00	—	20.00	9.40	15.00
	(Molar yield)		0.910		1.212	0.706	0.910
Lactic acid fonnd	(mM/dl)	—	11.00	10.00	7.00	11.10	3.60
	(Molar yield)		0.668	0.991	0.424	0.836	0.218
Pyruvic acid found	(mM/dl)	Trace	0.34	0.20	Trace	Trace	0
	(Molar yield)		0.021	0.020			
α-Ketoglutaric acid found	(mM/dl)	0	Trace	0	Trace	0	1.24
	(Molar yield)						0.075
Bacterial cells found	(mg/dl)	—	219	—	—	35	296

TABLE IV. INFLUENCE OF AGITATION AND AERATION UPON α-KETOGLUTARATE-FERMENTATION BY *E. coli*

Amounts of nitrogen added $(NH_4)_2SO_4$ (g/dl)	NH_4Cl (g/dl)	$(NH_4)_2HPO_4$ (g/dl)	Agitator speed (r. p. m.)	Aerating rate of air (vol per vol per min.)	Time of incubation (hours)	Glucose consumed (g/dl)	Pyruvic acid found (g/dl)	α-Ketoglutaric acid found (% on glucose consumed)
(with 10 liter-fermenter)								
0	0	0.10	60	1.0	46	2.90	0.41	7.9
0	0	0.15	60	1.0	37	3.89	0.77	Trace
0.05	0	0.10	100	0.35	47	2.83	0	31.7
0.10	0	0.10	160	1.0	50	4.46	0	50.3
0.10	0	0.10	160	1.0	38	2.48	0	54.5
0.10	0	0.10	170	1.0	42	3.95	0	56.2
0.10	0.10	0.10	180	1.0	39	4.15	0	55.0
0.20	0.10	0.10	180	1.0	109	10.47	0	32.4
0.20	0.10	0.10	180	1.0	87	8.80	0	35.5
0.20	0.10	0.10	180	1.0	108	10.55	0	38.7
(with 500ml-shaking flask)								
0	0	0.15	shaken on shaker		42	2.35	0	36.2
0	0	0.15	shaken on shaker		85	3.45	0	49.2
0.10	0	0.15	shaken on shaker		85	4.90	0	45.5
0.10	0	0.10	shaken on shaker		168	8.83	0	45.6

TABLE V. PRODUCTION OF PYRUVIC AND α-KETOGLUTARIC ACIDS FROM GLUCOSE BY *E. coli*

	Experiment I			Experiment II			
Time of incubation (hours)	14	24	37	14	20	36	46
Agitator speed (r. p. m.)	60	60	60	120	120	120	120
Aerating rate of air (vol per vol per min.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Glucose consumed (mg/ml)	15.6	30.3	38.9	20.0	28.7	40.3	44.4
Pyruvic acid found (mg/ml)	2.7	5.2	7.7	1.3	Trace	0	0
α-Ketoglutaric acid found (mg/ml)	Trace	Trace	Trace	1.4	7.0	16.9	19.0

Medium contained 5.4% glucose, 0.15% $(NH_4)_2HPO_4$, 0.05% $(NH_4)_2SO_4$ and other mineral salts.

TABLE VI. SYNTHESIS OF CELL OF *E.coli* IN EARLY STAGE OF α-KETOGLUTARATE-FERMENTATION

Time of incubation (hours)	Glucose consumed (g/dl)	Bacterial cells synthesized (g/dl)	α-Ketoglutaric acid found (g/dl)	Pyruvic acid found (g/dl)	Agitator speed (r.p.m.)	Aerating rate of air (V per V per min.)
22	2.330	0.339	1.088	0	180	1.0
37	2.990	0.380	1.535	0	180	1.0
22	1.52	0.257	0.405	0	100	1.5
38	2.15	0.368	0.897	0	100	1.5

(high efficiency of aeration).

Therefore, in the case of α-ketoglutarate-fermentation on a large scale (with a large fermentor), aeration must be accompanied with agitation of the medium; the resulting aeration-efficiencies depend upon both air-flow and agitation (see Table I).

Table IV shows the effects of agitator-speed and aeration-rate upon the oxidative fermentation of glucose by *E. coli*. Incubations were carried out in both aeration with air-flow and agitation by propellers. The media contained 3~11% glucose, the amounts of ammonium-nitrogen and other mineral matters as already mentioned. It is worth to note that a much lower yield of α-ketoglutaric acid was generally observed when

the accumulation of pyruvic acid was increasing (see Table V). α-Ketoglutaric acid-fermentation took place under the effective aeration rates of about 0.7~3.0 mM O_2 per liter per minute. Increasing the effective aeration rate from about 0.7 to 3.0 mM O_2 per liter per minute resulted in accelerating the fermentation rate. Table VI shows the cell-synthesis in *E. coli* inducing α-ketoglutarate-fermentation. It is evident that α-ketoglutarate-fermentation is always accompanied with the remarkable production of the bacterial cell, although the fate of glucose-carbon is greatly dependent upon the C/N value of a culture medium as previously reported. It may now be suggested that both pyruvate and α-ketoglutarate are the starting substance in the oxidative assimilation in microorganisms. Some workers have indicated the principal role of pyruvate in oxidative assimilation[13,19].

Manometric Experiments. From the results hitherto mentioned, the following facts have been found; if *E. coli* is incubated in the presence of glucose under such low levels of available oxygen as to give effective aeration of about 0.1~0.4 mM oxygen per liter per minute, there are the increasing amounts of acetate and bacterial cells against the decreasing amount of lactate, while α-ketoglutarate-fermentation takes place under high levels of oxygen (about 0.7~3.0 mM O_2 per L per min.). It is now of interest to ascertain the mode of oxidation of various substrates with a Warburg technique. Experiments were at first carried out with glucose in the following manner: the reaction mixture (2.5 ml) contained 100~500 μM phosphate buffer (pH: 5.6), 4 μM $MgSO_4$, 10~50 μM glucose and 10~40 mg washed cells of *E. coli* (G-2 strain) grown aerobically on glucose, and in the centre well, 0.2 ml H_2O or 15% KOH. Incubations were carried out at 30°C in air. The efficiency of aeration of the Warburg manometer employed here was ascertained to be about 0.15~0.35 mM oxygen per liter per minute. The results of experiments are given in Table VII, from which it will be seen that the bacterial cells oxidize most of glucose to carbon dioxide and water with a great readiness, but there is little or no formation of α-ketoglutarate to be observed. The complete oxidation and the oxidative assimilation of glucose may proceed according to the following equations[20,21]:

Complete oxidation

$$C_6H_{12}O_6+6O_2 \longrightarrow 6CO_2+6H_2O$$

Oxidative assimilation

$$C_6H_{12}O_6+3O_2 \longrightarrow 3(CH_2O)+3CO_2+3H_2O$$

Similar experiments were carried out with pyruvate; Warburg vessel contained 100~500 μM phosphate buffer (pH: 6.0) 4 μM $MgSO_4$, 10~40 mg washed cells of *E. coli* grown aerobically on glucose and the indicated

Table VII. Manometric Observation on Oxidation of Glucose by Washed Cells of *E. coli* Grown Aerobically on Glucose

Exp. No.	Glucose consumed (μM)	O_2 consumed (μM)	CO_2 evolved (μM)	α-Ketoglutaric acid found (μM)
I	10.0	42.5	41.5	0
II	10.0	35.5		0
III	10.0	46.5	43.0	0
IV	20.0	90.0	88.5	0
V	50.0	170.5		0.6

Incubation for 2~6 hours.

Table VIII. Manometric Observation on Oxidation of Pyruvate by Washed Cells of *E. coli* Grown Aerobically on Glucose

Exp. No.	Pyruvic acid added (μM)	Pyruvic acid consumed (μM)	O_2 consumed (μM)	CO_2 evolved (μM)	α-Ketoglutaric acid found (μM)
I	10.0	10.0	20.5	21.0	Trace
II	10.0	10.0	16.7	17.0	0
III	20.0	20.0	31.1	30.5	0.5
IV	60.0	60.0	87.5		1.5
V	100.0	100.0	130.5		2.5

Incubation for 2~8 hour.

amounts of Na-pyruvate, and in the centre well, 0.2 ml H_2O or 15% KOH and in the side arm, 0.2 ml 4N H_2SO_4; total volume, 2.5 ml; incubation at 30°C in air. Table VIII shows that the pyruvate is mainly oxidized to carbon dioxide and water, although a part of substrate-carbon may be assimilated by the organism. In the previous papers of this series, the authors have demonstrated that C_3-acids such as pyruvate and lactate are a potent precursor in the formation of α-ketoglutarate under the high levels of oxygen (also see Table I~III). It will, however, be seen in Table VIII that when the oxidation of pyruvate proceeds at such low levels of available oxygen that aeration is carried out with a Warburg-respirator, little or no formation of α-ketoglutarate occurs.

The bacteria of *coli-aerogenes* are well known to oxidize acetate to carbon dioxide and water[7,20~22]. The organism used in this work was also ascertained to reveal an exceedingly high ability of oxidizing acetate to carbon dioxide and water. The inhibiting effects of arsenite on the oxidation of pyruvate and acetate are shown in Table IX, in which experiments were carried out with

TABLE IX. INFLUENCE OF ARSENITE UPON OXIDATION OF PYRUVATE AND ACETATE BY WASHED CELL OF *E. coli* GROWN AEROBICALLY ON GLUCOSE

Substrates added (μM)	Na-arsenite added (M/l)	O_2 consumed (μM)
Pyruvate		
10.0	0	16.7
10.0	4×10^{-2}	1.8
Acetate		
10.0	0	15.5
10.0	4×10^{-2}	0

Each vessel contained 150 μM phosphate buffer (pH 6.2), 4 μM $MgSO_4$, 5 mg washed cells of *E. coli* (G- 2 strain) and stated amounts of Na-arsenite and substrates; total volume 2.5ml; incubation for 2 hours at 30°C in air.

a glucose-grown cell in the similar manner as already mentioned. Table X also shows the oxidative activities towards various substrates of *coli-aerogenes*. The equations for the complete oxidations and oxidative assimilations of several organic acids by washed cells of *E. coli* may be summarized according to Clifton et al.[20,21] as follows:

$$CH_3COOH+2O_2 \rightarrow 2CO_2+2H_2O$$
$$CH_3COOH+1.5O_2 \rightarrow 0.5(CH_2O)+1.5O_2+1.5H_2O$$
$$CH_3COCOOH+2.5O_2 \rightarrow 3CO_2+2H_2O$$
$$CH_3COCOOH+1.5O_2 \rightarrow (CH_2O)+2CO_2+H_2O$$
$$CH_3CHOHCOOH+3O_2 \rightarrow 3CO_2+3H_2O$$

TABLE X. OXIDATION OF VARIOUS ORGANIC ACIDS BY *Coli-aerogenes* BACTERIA

Bacteria used	Washed cells of *E. coli* grown aerobically on glucose-medium (Q_{O_2})	Dried cells of *E. coli* grown aerobically on acetate-medium (Q_{O_2})	Dried cells of *A.aerogenes* grown aerobically on citrate-medium (Q_{O_2})
Substrates			
Acetate	38	30	30
Pyruvate	50	45	40
DL-Lactate	60		
DL-Malate	65	48	55
Fumarate	55	40	48
Succinate	68	55	63
α-Ketoglutarate	Ground cell 6	5	7
Citrate	Ground cell 7	6	38

$$CH_3CHOHCOOH+2O_2 \rightarrow (CH_2O)+2CO_2+2H_2O$$
$$HOOC\,CH_2CH_2\,COOH+3.5O_2 \rightarrow 4CO_2+3H_2O$$
$$HOOC\,CH_2CH_2\,COOH+2.5O_2 \rightarrow (CH_2O)+3CO_2+2H_2O$$

DISCUSSION

It has frequently been observed by many workers that molecular oxygen which is available, produces a striking effect upon the mode of metabolisms of substrates in micro-organisms. In the case of respiration, it has been recognized that the presence of free oxygen is indispensable for the operation of respiratory system and the metabolic rate depends upon the amount of available oxygen. On the other hand, the tricarboxylic acid cycle is generally believed to be the most fundamental mechanism for the oxidation of acetic acid.

From the results of investigations mentioned both in previous and present papers, the authors have indicated the following important points. (1) A gentle aeration suppresses the formation of lactate but increases the production of acetate, carbon dioxide and the bacterial cell, when *E. coli* is grown in the presence of glucose. (2) When the bacterial cells of *coli-aerogenes* are incubated with a gentle aeration, i.e., under low levels of available oxygen, not only carbohydrates, C_3-, C_4- and C_6- acids but also C_2- acid (acetate) are mainly oxidized to carbon dioxide and water, while, α-keto-

glutarate-fermentation does not occur under the conditions[4~6]. (3) α-Ketoglutarate-fermentation with C_3- and C_4-acids occurs only at high levels of oxygen, i.e., under the conditions so that a large amount of oxygen is supplied. However C_6-tricarboxylic acids reveal an exceedingly low potency in the formation of this C_5-ketonic acid by the bacterial cells[6]. (4) The onset of oxidative assimilation (synthesis of bacterial cell) is attained under low levels of oxygen. Thus, α-ketoglutarate-fermentation actually occurs under the highest level of oxygen. (5) C_3- and C_4-acids are the most potent precursors in the formation of α-ketoglutaric acid, but C_2-acid (acetate) which is the oxidation-product of these substances is not the potent precursor when it is used as a sole source of carbon[4~1]. The addition of acetate to the C_3- or C_4-acids-containing media bring about an increase in the yield of α-ketoglutarate[6]. The inhibiting effect of a small amount of oxygen (low aeration) upon the production of α-ketoglutarate would, therefore, be explained as follows: both the oxidations of carbohydrates, C_3- and C_4-acids to C_2-acid (acetate) and the complete oxidation of acetate into carbon dioxide and water occur, with an extreme readiness, at a low level of oxygen, so that the presence of a small amount of oxygen causes the deficiency in the precursors in α-ketoglutaric acid-synthesizing reaction. It, moreover, appears that α-ketoglutaric acid—synthesizing enzyme system may be active under the high levels of oxygen. Consequently, it may be indicated by the authors that the terminal respiration in microorganism does not proceed only by way of the definite cycle, but the direction of aerobic respiration is, indeed, changeable according to variation of the environmental oxygen-level. Based on the experimental results mentioned in the previous and present papers, it may also be indicated that the following respiratory systems; the conventional tricarboxylic acid cycle, the cleavage-system of C_6-tricarboxylic acids, and the dicarboxylic acid cycle are operative even under the low levels of oxygen. Thus, the metabolisms of substrate-carbon in *coli-aerogenes* may be approximately summarized as follows:

(I) **Under anaerobic conditions**

(I–a) Lactic acid-fermentation

$$C_6H_{12}O_6 \xrightarrow{+\frac{1}{2}H_2O} CH_3CHOHCOOH + \tfrac{1}{2}CH_3COOH + \tfrac{1}{2}C_2H_5OH + CO_2 + H_2$$

Other fermentation-products; acetoin, 2,3-butyleneglycol, succinic and formic acids

(I-b) Fermentative assimilation of substrate-carbon

(II) **Under low level of oxygen**

(II-a) Acetic acid-fermentation

Glucose → lactic (Pyruvic) acid → acetic acid

(II-b) Oxidative assimilation of substrate-carbon

(II-c) Complete oxidation of substrate-carbon

$$C_6H_{12}O_6 \xrightarrow[\text{TCA cycle or DCA cycle}]{+6O_2} 6CO_2 + 6H_2O$$

$$\left.\begin{array}{l} C_6\text{-tricarboxylic acid} \\ C_4\text{-dicarboxylic acid} \end{array}\right\} \begin{array}{l} CH_3COCOOH \xrightarrow{+5O} 3CO_2 + 2H_2O \\ CH_3COOH \xrightarrow{+4O} 2CO_2 + 2H_2O \end{array}$$

(III) **Under high level of oxygen**

(III-a) α-Ketoglutaric acid-fermentation

$$C_6H_{12}O_6 \xrightarrow{+10/3\ O_2} {}^2/_3C_5H_6O_5 + {}^5/_3CO_2 + 4H_2O$$

$$\left.\begin{array}{l} C_4\text{-dicarboxylic acid} \\ \text{lactic acid} \end{array}\right\} \begin{array}{l} \rightarrow CO_2 + H_2O \\ CH_3COCOOH \xrightarrow{+\frac{1}{2}O_2} \\ \rightarrow \tfrac{1}{2}C_5H_6O_5 + \tfrac{1}{2}CO_2 + \tfrac{1}{2}H_2O \\ \rightarrow CO_2 + H_2O \end{array}$$

(III-b) Complete oxidation of substrate-carbon

(III-c) Oxidative assimilation of substrate-carbon

(IV) **Oxidative fermentation in the presence of inhibitors**

(IV-a) Pyruvic acid-fermentation

$$C_6H_{12}O_6 \xrightarrow[+As]{+8/3O_2} {}^4/_3C_3H_4O_3 + 2CO_2 + {}^{10}/_3H_2O$$

(IV-b) Acetic acid-fermentation

$$CH_3COCOOH \xrightarrow[\alpha,\alpha'\text{-dipyridyl or } o\text{-phenanthroline}]{+O} C_2H_4O_2 + CO_2$$

Results hitherto mentioned may throw some light on the problem of the evolution of metabolic pattern in living organism.

Acknowledgement. The authors wish to express their gratitude to Mr. T. Ito, Mr. Y. Ichikawa and Mr. T. Tsunoda for many helpful discussions and for willing and conscientious assistance. This investigation has also been supported in part by the Grant in Aid for Developmental Scientific Research defrayed from the Ministry of Education and by Ajinomoto Co., Inc. to which the authors' thanks are due.

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Studies on Oxidative Fermentation

Part XIX. Catabolism of K-5-Ketogluconate by *Acetobacter suboxydans*. II*

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Oxidation products of 5-ketogluconic acid by intact cells of *Acetobacter suboxydans* ATCC 621 were investigated further. Besides acetic, succinic, α-ketoglutaric and glycolic acids reported previously, a little amount of pyruvic acid was newly recognized in the oxidation products of K-5-ketogluconate. Members of the tricarboxylic acid cycle except oxalacetic acid, and acetic, L-tartaric and glycolic acids were not oxidized, while glycolaldehyde was oxidized quantitatively to glycolic acid by this organism. From these results, oxidation pathway of 5-ketogluconic acid was discussed.

It is well known that glucose is oxidized to 2-ketogluconic acid (2-KGA), 5-ketogluconic acid (5-KGA) or both of them via gluconic acid by *Acetobacter* and allied oxidative bacteria. It is considered to be important to clarify the further oxidative pathways of these ketogluconic acids. Several papers[1~6] have appeared on the metabolic route of 2-KGA, however, little attention has been paid to the oxidative route of 5-KGA. The authors studied this problem to elucidate metabolic pathway of 5-KGA and in the previous paper[7] it was ascertained that 5-KGA was oxidized by intact cell suspension of *Acetobacter suboxydans* ATCC 621, and acetic, succinic, glycolic acids and α-ketoglutaric acid (α-KGA) were confirmed as the oxidation products. The experiments in this paper were carried out in order to detect pyruvic acid, (supposed to be a precursor of acetic, succinic acids and α-KGA) and to clarify the roles of these organic acids in metabolism of 5-KGA.

* This work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan, Tokyo, on April 11, 1957.

1) H. Katznelson, S. W. Tanenbaum and E. L. Tatum, *J. Biol. Chem.*, **204**, 43 (1953).

2) J. De Ley, *Enzymologia*, **16**, 99 (1953).

3) J. De Ley, *ibid.*, **17**, 55 (1954).

4) J. De Ley and J. Vandamme, *J. Gen. Microbiol.*, **12**, 162 (1955).

5) K. Aida, M. Fujii and T. Asai, This Bulletin, **21**, 30 (1957).

6) M. Ameyama and K. Kondo, *ibid.*, **22**, 380 (1958).

7) T. Asai, H. Murooka and Y. Kobayashi, *J. Agr. Chem. Soc. Japan*, **31**, 225 (1957).

EXPERIMENTAL

1. Organism. *Acetobacter suboxydans* ATCC 621 was used. This strain was maintained for a long time in this laboratory on Koji extract agar slants containing $CaCO_3$.

2. Preparation of intact cell suspension and dried cells.

The methods used have been described previously[7]. The cells grown on glucose yeast extract agar medium containing $CaCO_3$ were harvested and washed three times with distilled water and resuspended in distilled water. Dried cells were prepared by drying the washed cells *in vacuo* on silica gel.

3. Reaction method. The reaction mixtures containing 50 ml of solution (a) or (b) in 500 ml shake flasks were incubated aerobically on a reciprocating shaker at 30°C. Samples were withdrawn periodially and allowed to analyses after centrifugation at 6,000 r.p.m. for 15 minutes in order to remove the cells.

Solution (a)

Intact cell suspension (dry weight 80 mg)	20 ml
M/10 Phosphate buffer solution (pH 5.0)	20 ml
M/4 K-5-Ketogluconate (K-5-ketogluconate, 348.3 mg)	6 ml
M/100 Glucose (glucose, 0.7 mg)	4 ml
Total volume	50 ml

Solution (b)

Intact cell suspension (dry weight 80 mg)	20 ml
M/10 Phosphate buffer solution (pH 5.0)	20 ml
M/100 Glucose (glucose, 0.7 mg)	4 ml
Distilled water	6 ml
Total volume	50 ml

4. Qualitative and quantitative determination of keto acids.

Keto acids and other carbonyl compounds were identified by means of paper chromatography after the method of Cavallini, Frontali and Toschi[8]. The developing solvent was *n*-butanol saturated with 3% NH_4OH in water and papers (Tôyô filter paper, No. 50) were developed by ascending method at room temperature. The estimation of keto acids was performed by the Shimizu's modification[9] of Friedemann-Haugen's method.

5. Oxygen uptake and carbon dioxide evolution were determined by means of Warburg manometer at 30°C in air. Reducing power was determined by the Somogyi's method modified by Kobayashi and Tabuchi[10]. The spectrophotometric method of Calkins[11] was applied for the estimation of glycolic acid. Absorption spectra of 2,4-dinitrophenylhydrazone of keto acids were obtained with a Beckman DK-2 type spectrophotometer.

6. Materials. The crystalline K-5-ketogluconate used as the substrate was prepared from Ca-5-ketogluconate. Glycolaldehyde synthesized from tartaric acid by the method of Fischer and Taube[12] was supplied from Dr. Nobuo Ikekawa of this Institute. The other sodium salts of organic acids and free acids were obtained commercially. The free acids were neutralized with NaOH before use.

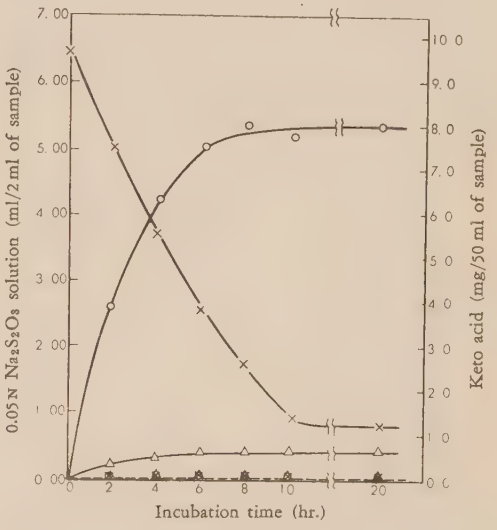

FIG. 1. Periodical Changes in Amount of Keto Acids produced from K-5-Ketogluconate.

—— Solution (a) ---- Solution (b)
○ α-Ketoglutaric acid
△ Pyruvic acid × Reducing sugar

RESULTS

1. Periodical changes in the amount of keto acids produced from K-5-ketogluconate.

The curve (1) in Fig. 1 shows the periodical changes in the amount of xylol insoluble compounds represented as α-KGA. α-KGA was identified as a reaction product in the previous paper[7]. The curve (2) shows xylol soluble 2,4-dinitrophenylhydrazone* expressed as pyruvic acid.

8) D. Cavallini, N. Frontali and G. Toschi, *Nature*, **163**, 568 (1949).
9) T. Shimizu, *J. Biochem.*, **37**, 421 (1950).
10) T. Kobayashi and T. Tabuchi, *J. Agr. Chem. Soc. Japan*, **23**, 171 (1954).
11) V.P. Calkins, *Anal. Chem.*, **15**, 762 (1943).
12) H.O.L. Fischer and C. Taube, *Ber.*, **60**, 1707 (1927).

As seen in the figure, the xylol insoluble compounds (2,4-dinitrophenylhydrazone of α-KGA) and the xylol soluble compounds (2, 4 - dinitrophenylhydrazone of pyruvic acid) increased with decreasing reducing sugar in the reaction mixture.

The amount of the xylol insoluble compounds increased and reached to the maximum after 8 hours incubation. Namely, 7.95 mg of the xylol insoluble compounds (estimated as α-KGA) corresponding to about 3 per cent of 5-KGA supplied was accumulated in 50 ml of the reaction mixture, while the xylol soluble compounds increased at the same time and the maximum quantity (674γ estimated as pyruuic acid), was gained after 6 hours incubation.

Estimated values of xylol insoluble and soluble compounds on the solution (b) were negligible. These results showed that reaction products with 2,4-dinitrophenylhydrazine were not due to a little amount of added glucose or to the cell materials.

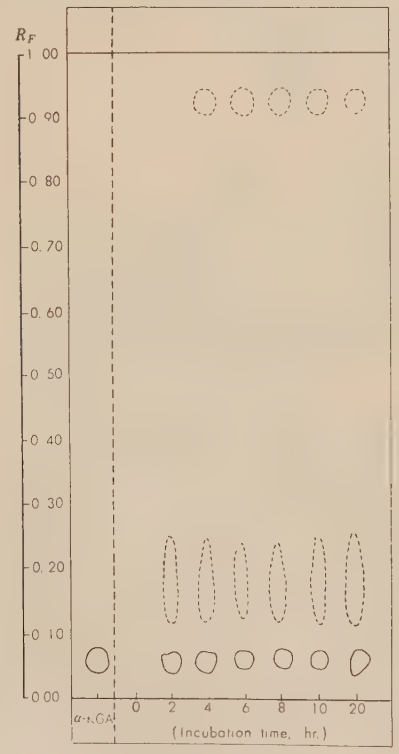

FIG. 2. Paperchromatogram of Xylol Insoluble Fraction of 2,4-Dinitrophenylhydrazones.

* It is possible to contain other reaction products than the 2,4-dinitrophenylhydrazone of pyruvic acid.

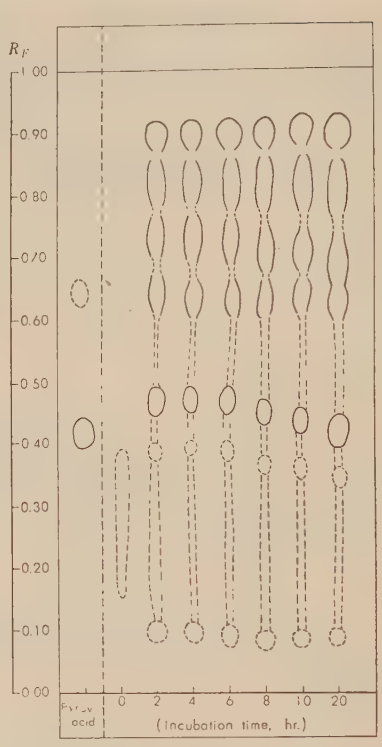

FIG. 3. Paperchromatogram of Xylol Soluble Fraction of 2,4-Dinitrophenylhydrazones.

In order to clarify the nature of the reaction products with 2,4-dinitrophenylhydrazine, the xylol insoluble and soluble compounds were investigated by means of paperchromatography on each portion of fluid withdrawn from the reaction mixture at the different time intervals. The results were shown on the chromatograms in Fig. 2 and Fig. 3.

As shown in Fig. 2, the chromatogram of the xylol insoluble compounds gave three spots, one was a thick spot with R_F value 0.06~0.07, coinciding with the spot of 2,4-dinitrophenylhydrazone of α-KGA and other two were very thin with R_F values 0.93 and about 0.20 (tailing spot). It is clear that the xylol insoluble phenylhydrazone is almost derived from that α-KGA. As shown in Fig. 3, the chromatograms of the xylol soluble compounds gave five thick spots with R_F values 0.42~0.47, 0.63~0.64, 0.73~0.76, 0.81~0.82, 0.90 (the latter four spots were connected together). The spot with R_F value 0.42~0.47 was probably that of 2,4-dinitrophenylhydrazone of pyruvic acid compared with

the phenylhydrazone of pure authentic pyruvic acid which gives two spots, a thick with R_F 0.42 and a thin R_F 0.64. The other spots excepting the spot of R_F 0.63~0.64 were distinctly different from those of phenylhydrazone of pyruvic acid. The nature of these compounds will be reported later.

2. Identification of pyruvic acid

400 ml of solution (a) described above, divided into eight 500 ml shake flasks (each flask contained 50 ml of the solution) was aerobically incubated 30°C on a reciprocating shaker for 6 hours. After reducing power in the reaction mixture decreased to 50~55%, reaction was ceased and the solution was centrifuged to remove the cells. The supernatant was added with 100 ml of 2,4-dinitrophenylhydrazine solution in HCl and allowed to stand at room temperature for 30 minutes. The solution was extracted with xylol. The 2,4-dinitrophenylhydrazones were re-extracted with 10 per cent $NaHCO_3$ solution, acidified immediately with HCl solution (1 : 1), and finally extracted with ethyl acetate. Ethyl acetate was evaporated and the residue was again dissolved in a small quantity of ethyl acetate and it was spotted in line 2.5 cm far from the edge of a filter paper (Tôyô filter paper No. 50, 40×40 cm). The paper was developed by the method described above. After developing and drying, the part of the paper corresponding to the spot of 2,4-dinitrophenylhydrazone of pyruvic acid was cut off and extracted with 10 per cent $NaHCO_3$ solution, acidified at once with HCl solution, re-extracted with ethyl acetate and finally ethyl acetate was evaporated. The paperchromatogram of this residual substance gave the spots identical to those of the 2,4-dinitrophenylhydrazone of pure authentic pyruvic acid. And then the thick spot was cut off and eluted with 10 per cent Na_2CO_3 solution. The absorption spectrum of this solution obtained through a Beckmon DK-2 type spectrophotometer, showed the same figure and the same maximum absorption peak as those of the 2,4-dinitrophenylhydrazone of authentic pyruvic acid. The absorption curve obtained after the above solution was alkalinized by adding the equal quantity of 1.25 N NaOH solution, showed also the same figure and the same maximum absorption peak as those of pure authentic pyruvic acid treated similarly. Thus, it was ascertained that the compound with R_F value 0.42~0.47 was pyruvic acid. These resuls were shown in Table 1 and Fig. 4.

TABLE I. MAXIMUM ABSORPTION OF 2,4-DINITROPHENYLHYDRAZONES OF PYRUVIC ACID AND THE SAMPLE.

	Solvent	Pyruvic acid (mμ)	Sample (mμ)
(a)	10% Na_2CO_3	370	370
(b)	10% Na_2CO_3+1.25 N NaOH	446	446

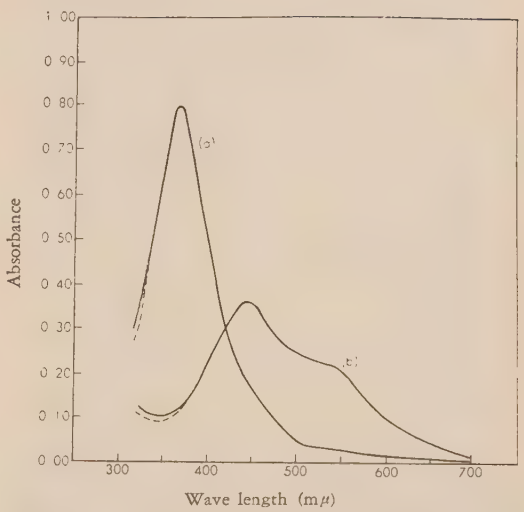

FIG. 4. Absorption Spectrum of 2,4-Dinitrophenylhydrazones of Pyruvic Acid and the Sample.
(a) Solvent: 10% Na_2CO_3
(b) Solvent: 10% Na_2CO_3+1.25 N NaOH
—— Sample
---- Standard

3. Oxidation of the members of tricarboxylic acid cycle and acetic acid.*

As reported previously[7], 5-KGA was oxidized by intanct resting cell suspension of *A. suboxydans* and a little amount of succinic acid and α-KGA were formed and moreover, in this experiment pyruvic acid was also detected in the reaction mixture as described above. These results suggest that 5-KGA may be oxidized to pyruvic acid and to CO_2 and H_2O via tricarboxylic acid cycle. However, dried cells of this bacteria, which are able to oxidize actively 5-KGA did not oxidize several members of tricarboxylic acid cycle, namely fumaric, succinic, malic, citric, oxalosuccinic acids and α-KGA, except oxalacetic acid. Acetic acid detected in the oxidation products of 5-KGA was not also attacked by

* As reported previously[13], addition of a little amount of glucose was necessary for the oxidation of 5-KGA by this organism, but sometimes it could be oxidized without glucose and even in this case, accelerative effect of glucose was observed. The dried cells used in this experiment were the cells capable of oxidizing 5-KGA without glucose.

13) T. Asai and H. Murooka, *J. Agr. Chem. Soc. Japan*, **30**, 1 (1956).

TABLE II. OXIDATION OF THE MEMBERS OF TRICARBOXYLIC ACID CYCLE, ACETIC AND GLYCOLIC ACIDS BY DRIED CELL SUSPENSION OF *A. suboxydans*.

Substrate	Oxygen uptake at 180 minutes (μl)
5-Ketogluconate	259.0
Citrate	10.2
Oxalosuccinate	9.5
α-Ketoglutarate	12.5
Succinate	8.0
Fumalate	16.3
Malate	17.1
Oxalacetate	42.0
Acetate	9.1
Glycolate	11.2
Endogenous	8.3

The system contained: 0.8 ml M/10 phosphate buffer (pH 5.0), 5 μM substrate (as sodium salt) and 30 mg dried cells. Total volume 2.0 ml. 0.2 ml 20% KOH in center well. Incubated at 30°C in air.

the dried cells of this organism (Table II).

4. Oxidation of glycolaldehyde and glycolic acid.

Intact cells of *A. suboxydans* oxidized glycolaldehyde without CO_2 evolution (Fig. 5). The amount of O_2 uptake was 1/2 mole for one mole of the substrate supplied. Paperchromatogram of this reaction mixture gave only one spot which being identical with that of authentic glycolic acid with R_F value 0.57. Estimation of glycolic acid by the Calkin's method showed that 95% of glycolaldehyde changed to glycolic acid. Glycolic acid, one of the oxidation products from 5-KGA as reported in the previous paper[7], was not attacked further (Table II). These results agreed with the experiment reported recently by Fewster[14].

5. Oxidation of L-tartaric acid.

According to R. W. Jackson et al.[15], L-tartaric acid was produced from K-5-ketogluconate by *Pseudomonas fluorescens*, but it was not found in the oxidation products from the same substrate by *A. suboxydans*[7]. However, if L-tartaric acid formed was rapidly decomposed by the cells, this acid might not be accumulated in the reaction mixture. Thus, it was examined whether or not L-tartaric acid is oxidized by the dried cells. As shown in Table III, L-tartaric acid was not attacked and this result supports Fewster's[14] recent report, demonstrating that D-, L-, and *meso*tartaric acids were not

14) J. A. Fewster, *Biochem. J.*, **69**, 582 (1958).

15) R. W. Jackson, H. J. Koepsell, L. B. Lockwood, G. N. Nelson and F. H. Stodola, Inter. Congr. Biochem. Abstrs. of Communs. 1st Congr., Cambridge Engl. 1949, 536-7; *Chem. Abst.*, **47**, 2260 (1953).

TABLE III. OXIDATION OF L-TARTARIC ACID BY DRIED CELL SUSPENSION OF *A. suboxydans*.

Substrate	Reaction time (min.)	Oxygen uptake (μl)
5-Ketogluconate (50 μM)	90	95.5
L-Tartarate (50 μM)	90	7.0
Endogenous	90	2.4
5-Ketogluconate (50 μM) +glucose (0.2 μM)	40	215.7
Glucose (0.2 μM)	40	12.6
L-Tartarate (50 μM) +glucose (0.2 μM)	90	17.8
Glucose (0.2μM)	90	15.0

The system contained: 0.8 ml M/10 phosphate buffer (pH 5.0), 30 mg dried cells in 0.8 ml distilled water, 0.2 ml 50 μM K-5-ketogluconate or K-L-tartarate (or distilled water) and 0.2 ml 0.2 μM glucose (or distilled water). Total volume 2.0 ml. 0.2 ml 20% KOH in center well. Incubated at 30°C in air.

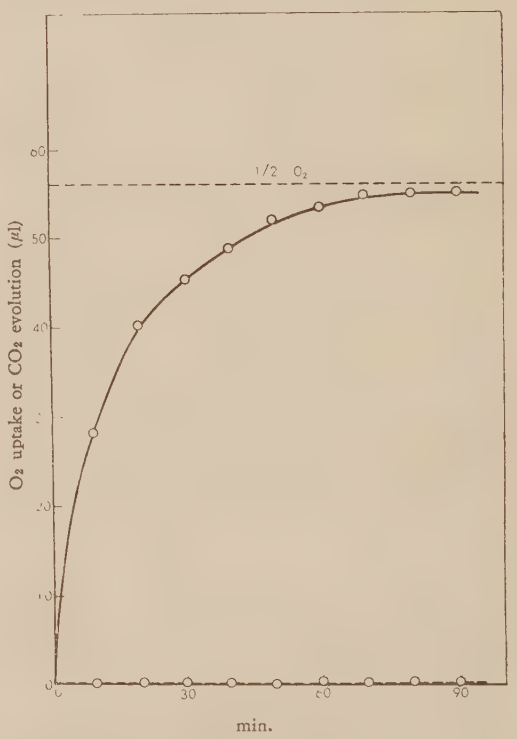

FIG. 5. Oxidation of Glycolaldehyde by Intact Cell Suspension of *A. suboxydans*.

The system contained: 0.8 ml M/10 phosphate buffer (pH 5.0), 5 μM glycolaldehyde in 0.7 ml distilled water and 0.5 ml cell suspension (2 mg dry weight). Total volume, 2.0 ml. 0.2 ml 20% KOH in center well. Incubated at 30°C in air.

—— O_2 uptake
---- CO_2 evolution

oxidized by the washed cells of *A. suboxydans*. Even a little amount of glucose was added in the reaction system in which 5-KGA was oxidized actively, L-tartaric acid was also not attacked. These results suggest strongly that this acid may not be an intermediate of 5-KGA oxidation.

DISCUSSION

Metabolic pathway of K-5-ketogluconate was investigated by *A. suboxydans* and it was reported in the previous paper[7] that a little amount of acetic, succinic, glycolic acids and α-KGA were accumulated as oxidation products. Succinic acid and α-KGA were known as members of the tricarboxylic acid cycle in which pyruvic acid is a precursor of these organic acids. The results of the present experiments showed the presence of pyruvic acid as the oxidation product of 5-KGA. Thus, it was presumed that 5-KGA was converted to pyruvic acid and the latter was oxidized to CO_2 and water via tricarboxylic acid cycle. If this pathway was the case, each organic acid belonging to the members of tricarboxylic acid cycle should be oxidized. However, the results of the present experiments showed that fumaric, succinic, malic, citric, oxalosuccinic acids and α-KGA except oxalacetic acid were not attacked at any extent by dried cells of this organism while 5-KGA was oxidized actively. The experiments with intact cells[16] and cell-free extracts[17] by King et al. suggested that the presence of tricarboxylic acid cycle is questionable. In accordance with these experiments, it might be probable that succinic acid and α-KGA were produced via another pathway than the route of tricarboxylic acid cycle. King et al.[18] reported the formation of acetic acid from pyruvic acid by *A. suboxydans* and they confirmed that pyruvic acid was at first decarboxylated to acetaldehyde and then the latter was oxidized to acetic acid. Since in our present experiment pyruvic acid was found besides acetic acid in the oxidation products of 5-KGA, it may be presumed that the substrate degraded to pyruvic acid by an unknown pathway and the latter was metabolized to acetic acid through the mechanism mentianed above. Acetic acid produced was not oxidized further by dried cells as shown in Table II. Kitos et al.[19] also ascertained that acetic acid was not oxidized either by whole cells, cell free extract, or cell free homogenates of this organism while the cell free extract catalyzed the formation of acetyl-CoA and acetate was incorporated in small amounts into cell lipid fractions, only in the presence of an energy source. Attempts to detect the condensing enzyme and the route of further degradation of acetate were unsuccessful. Jackson et al.[15] confirmed that L-tartaric, glycolic, formic acids and CO_2 were produced from K-5-ketogluconate by *P. fluorescens*. From this result it is presumed that 5-KGA is degraded to tartaric semialdehyld and glycolaldehyde after enolization and cleavage between carbon 4 and carbon 5 of this acid, and that tartaric and glycolic acids are formed through the oxidation of these products. The results of our present experiments by *A. suboxydans* showed that glycolaldehyde was oxidized quantitatively to glycolic acid, however, glycolic and tartaric acids were not oxidized at all, only a little amount of glycolic acid was formed from 5-KGA, and L-tartaric acid was not so far detected in the oxidation products. These facts suggest that although the hypothetical scheme of 5-KGA breakdown mentioned above is a probable one in the case of oxidation by *P. fluorescens*, it may not play a significant role in the oxidation of 5-KGA by *A. suboxydans* if it is present. It seems likely that 5-KGA is converted to 5 carbon compound and oxidized to CO_2 and water via pentose cycle, which was ascertained by Hauge et al.[20] to be operative in the carbohydrate oxidation system of *A. suboxydans*.

16) T.E. King and V.H. Cheldelin, *J. Biol. Chem.*, **220**, 177 (1956).
17) T.E. King and V.H. Cheldelin, *Biochem. Biophys. Acta*, **14**, 108 (1954).
18) T.E. King, C.J. Stewart and V.H. Cheldelin, *J. Biol. Chem.*, **208**, 821 (1954).
19) P.A. Kitos, T.E. King and V.H. Cheldelin, *J. Bact.*, **74**, 565 (1957).
20) J.G. Hauge, T.E. King and V.H. Cheldelin, *J. Biol. Chem.*, **214**, 1 (1955).

Concerning the initial reaction of 5-KGA breakdown, J. De Ley[21] suggested the formation of 6-phospho-5-ketogluconic acid from 5-KGA by *Klebsiella* and it was supposed also from our further experiments that the first step of 5-KGA oxidation might be an activation reaction of this acid, as the oxidation of this acid was accelerated with the addition of ATP. Details of this problem will be reported in the next paper.

SUMMARY

Further investigations on the oxidation products and the oxidative pathway of 5-ketogluconic acid by intact and dried cells of *Acetobacter suboxydans* ATCC 621 gave the following results.

(1) The yield of α-ketoglutaric acid was about 3% of 5-ketogluconic acid supplied as the substrate and the former acid produced did not undergo further oxidation.

(2) Besides acetic, succinic, α-ketoglutaric and glycolic acids as reported in the previous paper, a little amount of pyruvic acid was found in the oxidation products by this organism.

(3) It was ascertained that dried cells of this organism which were capable of oxidizing 5-ketogluconic acid did not oxidize the members of the tricarboxylic acid cycle and related compounds except oxalacetic acid. This result suggests that tricarboxylic acid cycle may not be operative in the degradation processes of 5-ketogluconic acid.

(4) Glycolaldehyde was oxidized by this organism quantitatively but glycolic acid produced could not be oxidized further.

(5) L-Tartaric acid could not be oxidized even though a little amount of glucose was added into the reaction system.

Acknowledgement Authors wish to thank Dr. Nobuo Ikekawa of this Institute for the synthesis and the supply of glycolaldehyde and Mr. Masataka Yamazaki, Fujisawa Pharmaceutical Co., Ltd. for the supply of Ca-5-ketogluconate.

21) J. De Ley, *Biochim. Biophys. Acta*, **27**, 652 (1958).

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Biosynthesis of Carotene in Disks of Carrot Root*

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Net synthesis of carotene was demonstrated in disks obtained from carrot roots and grown in test tubes on media containing fructose, glucose or sucrose. Mannitol showed no effect on both growth of the disks and carotene synthesis. Diphenylamine inhibited the synthesis of carotene in the disks in a way somewhat different from that in the case of microorganisms. Although yeast extract stimulated growth of the disks, it showed no effect on carotene synthesis. Addition of possible precursors for carotene synthesis to the medium showed no stimulatory effect except when β-hydroxy-β-methylglutaric acid was added to the medium rich in sucrose.

INTRODUCTION

Several years ago Ezell and Both[1] reported some increase in the amount of β-carotene contained by roots of carrot or of sweet potato during the storage. Meanwhile Goodwin[2] could not demonstrate the synthesis of polyenes in carrot roots grown in tissue culture. However, recently, he[3] showed the incorporation of mevalonic acid into the carotene molecule with carrot slices. Therefore, carrot roots, even after harvesting, appear to contain a more or less intact enzyme responsible for synthesizing carotene from other materials. This paper reports the net synthesis of carotene in disks obtained from carrot roots and grown in tissue culture with some observations pertaining to the biosynthesis of carotene in this system.

EXPERIMENTAL

Determination of carotene: Mackinney and Fratzke's method[4] for determining carotenes in dried carrot was adopted with some modifications. Several disks were homogenized with sand in a mortar and carotene was extracted from the homogenate with several 1.5 to 2 ml portions of acetone. Ten to 15 ml in total of acetone was required to extract carotene completely. The extracts were collected in a glass-stopperred test tubes and 10 ml of *n*-hexane was added to the combined extract. After gentle stirring of the mixture, it was washed with 10 to 15 ml (the same volume as that of acetone used for extracting carotene) of distilled water. The yellow solution of carotene in *n*-hexane thus obtained was transferred into another test tube and washed again with 10 ml of distilled water. The light absorbancy of this solution was measured at 450 mμ with Hitachi's spectrophotometer. The total amount of carotenes in the disks was calculated on the basis of β-carotene using the extinction coefficient: 2580[5]. Because α-carotene gives almost the same extinction as that of β-carotene and the amount of carotenoids other than α- and β-carotenes in carrot was very small, chromatographic separation was not necessary to determine the total amount of carotenoids. Considerable amount of ζ-carotene was present in the extract. However, it showes no measurable light absorption at 450 mμ. Light absorbancy at 430 mμ was also determined to estimate the total amount of polyenes including ζ-carotene. In this report, however, the results including the amount of ζ-carotene are not described, because the changes of the amount of total polyenes including ζ-carotene were essentially same as

* Parts of this report were presented at the meetings of the Agricultural Chemical Society of Japan, Tokyo, April 9, 1957 and Kyoto, May 4, 1958.

1) B.D. Ezell and M.S. Wilcox, *Food Res.*, **13**, 203 (1948); V. H. Booth, *J. Sci. Food Agr.*, **2**, 353 (1951).

2) T. W. Goodwin, *J. Sci. Food Agr.*, **4**, 209 (1953).

3) G. D. Braithwaith and T. W. Goodwin, *Biochem. J.* **61**, 13; **66**, 31 (1957).

4) G. Mackinney and W. E. Fratzke, *Anal. Chem.*, **19**, 614 (1947).

5) F.P. Zscheile, J.W. White Jr., B.W. Beadle and J.R. Roach, *Plant Physiol.*, **17**, 331 (1942).

TABLE I. COMPOSITION OF THE BASAL MEDIUM

	g/l		mg/l		mg/l
KNO_3	0.72	$B(OH)_3$	0.1	glycine	45
NaCl	0.42	$MnCl_2 \cdot 4H_2O$	0.9×10^{-2}	thiamine hydrochloride	1.5
KCl	0.22	$ZnSO_4 \cdot 7H_2O$	0.1		
$NaH_2PO_4 \cdot 2H_2O$	0.14	KI	1.0×10^{-3}	pyridoxine hydrochloride	2
$CaCl_2 \cdot 2H_2O$	0.075	$CuSO_4 \cdot 5H_2O$	3.0×10^{-3}	niacinamide	7.5
$MgSO_4 \cdot 7H_2O$	0.25	$NiSO_4 \cdot 6H_2O$	3.4×10^{-3}		g/l
		$Al(NO_3)_3 \cdot 9H_2O$	8.4×10^{-3}	dried yeast	1
		$FeSO_4 \cdot 7H_2O$	1.0	agar	10

those of carotenoids other than ζ-carotene. The results obtained by this method were fairly consistent with those obtained by extracting the pigments with methanol and benzene and saponifying the extract with alcoholic potassium hydroxide as described by Fujita[6].

Material and culture: A series of cores of 6 mm diameter including the cambium layer was removed from a carrot root purchased from the market with a sterile cork-borer, as described by White[7]. These cores were cut transversely into disks of 1 mm thickness. After two days' preincubation on the mannitol-medium, the compostion of which is described in the following section, these disks were transferred to the culture media, which were solidified aslant with agar in 15 ml portions in test tubes of 20 mm diameter and 20 cm length. Three to 5 disks were cultured in each tube, its opening being covered with tin foils. The test tubes with these disks were then incubated at 25°C for about fifteen days in the dark. Fresh weights and carotene contents of these disks were determined at intervals for each 3 to 5 disks in a test tube.

β-Hydroxy-β-methylglutaric acid (m. p. 105~108°C) was synthesized by the ozonolysis of diallylmethylcarbinol as described by Klosterman[8] with following modifications: ozonization product was oxidized with silver oxide in alkaline aquous medium and extracted with ethylether. β-Hydroxy-β-methylglutaric acid thus produced was recrystallized from a mixture of dioxane and benzene (1:8). β-Methylcrotonic acid (m. p. 66°C) was synthesized from mesityloxide by oxidation with chlorine in alkaline solution[9]. *d*, *l*-Mevalonic acid was kindly given by Assistant Prof. S. Tamura.

Gulture media: Composition of the basal medium was given in Table I. Heller's[10] indication was followed for inorganic nutrients, and White's[7] indication for organic nutrients other than yeast extract. Concentrations of organic micro nutrients were 1.5 times as much as those proposed by White. For carotene synthesis, 20 g of sucrose was included per liter of the basal medium. For preincubation, 10.7 g of mannitol was used in place of 20 g of sucrose. All components were dissolved in glass distilled water, and autoclaved at 14~16 lbs/inch^2 for ten minutes.

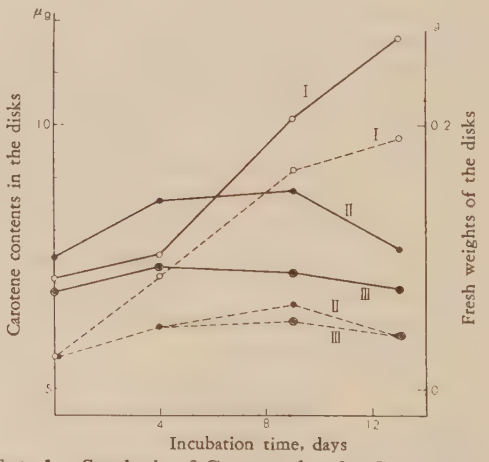

FIG. 1. Synthesis of Carotene by the Carrot Disks.

Solid lines represent changes of carotene contents in the disks, and dotted lines represent changes of fresh weights of the disks.
Lines I: Changes of carotene contents and fresh weights of the disks, cultured on the 2% sucrose media.
Lines II: Cultured on the no sucrose media.
Lines III: Cultured on the mannitol media.

6) A. Fujita, "Vitamin Teiryo-ho (Method of Vitamin Assay)," Nanko-do Co., 1955, p. 189.
7) P.R. White, "Cultivation of Animal and Plant Cells", The Donald Press Co., 1954, p. 71, 149.
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9) L. I. Smith, W. W. Prichard and L. J. Spillane, *Organic Synthesis, Collected Volume*, **3**, 302 (1955).
10) R. Heller, *Annales des Sciences Naturelles Botanique et Végétale. 11e série*, **XIV**, 1 (1953).

TABLE II. SYNTHESIS OF CAROTENE IN THE PRESENCE OF VARIOUS CARBON SOURCES

	sugars	fresh weights of the disks*		carotene contents in the disks*	
before incubation	——	0.073 g	(100)	4.45 μg	(100)
after incubation	no sugar	0.087	(120)	5.22	(118)
	mannitol	0.078	(107)	4.96	(112)
	glucose	0.128	(176)	6.26	(141)
	fructose	0.137	(188)	6.81	(153)
	sucrose	0.128	(176)	6.26	(141)

* Values of fresh weights or of carotene contents obtained before or after incubation of the disks weighing 0.073 g and containing 4.45 μg of carotene for fourteen days. Figures in brackets represent relative values, the initial values being taken as 100.

TABLE III. RELATIONSHIP BETWEEN THE CONCENTRATION OF SUCROSE IN THE MEDIUM AND THE AMOUNT OF CAROTENE SYNTHESIZED BY THE CARROT DISKS.

	sucrose concentration in the medium	fresh weights of the disks*		carotene contents in the disks*	
before incubation	——	0.115 g	(100)	6.79 μg	(100)
after incubation	0%	0.139	(121)	8.50	(125)
	0.25	0.178	(155)	9.05	(134)
	0.5	0.191	(166)	8.91	(131)
	1.0	0.234	(203)	10.24	(151)
	2.0	0.249	(217)	10.32	(152)
	4.0	0.253	(220)	11.42	(169)

* Values of fresh weights or of carotene contents obtained before or after incubation of the disks weighing 0.115 g and containing 6.79 μg of carotene for fourteen days. Figures in brackets represent relative values, the initial values being taken as 100.

RESULT

Synthesis of carotene by carrot disks: Figure 1 shows that the disks which were cultured on the sucrose media synthesized a considerable amount of carotene during growth. The amount of carotene synthesized in the experimental period (twelve to fifteen days) was usually about 50% of that contained in the disks before incubation. Sometimes the amount of carotene *de novo* synthesized reached 80~90% of that found initially. Table II shows the synthesis of carotene on media containing mannitol, fructose or glucose at concentrations equivalent in C-contents to 2% sucrose. While mannitol was poor as carbon source, glucose and fructose were utilized for both the carotene synthesis and growth of the disks as well. Fructose seemed to be more excellent as carbon source than sucrose or glucose. However, sucrose was used throughout this experiment.

Concentration of sucrose: The effect of sucrose concentration on the carotene synthesis is shown in Table III. In Table IV, results of two series of experiments are shown, which was designed to test the possible effect caused by the changes in the tonisity of the culture medium on the carotene synthesis.

The disks were cultured on media containing 2%, 0.5%, or no sucrose. In experiment A, the tonisities of the media were not controlled, but in experiment B, the tonisities were controlled by the addition of mannitol to the low-sucrose media. The amounts of carotene synthesized by the disks on the 0.5% sucrose-media were almost the same in experiments A and B. Addition of 1.07% mannitol (equimolar to 2% sucrose) to

Table IV. Effects of Additions of Yeast Extract and of Mannitol to the Medium on the Formation of Carotene in the Carrot Disks

		concentrations of sucrose	concentrations of mannitol	addition of yeast extract	fresh weights of the disks*		carotene contents in the disks*	
A	before incubation	——	——	——	0.113 g	(100)	4.19 μg	(100)
	after incubation	0 %	0 %	−	0.117	(104)	4.49	(107)
				+	0.135	(119)	4.97	(119)
		0.5	0	−	0.162	(144)	5.63	(134)
				+	0.229	(203)	6.11	(146)
		2.0	0	−	0.191	(169)	5.94	(142)
				+	0.266	(236)	6.62	(158)
B	before incubation	——	——	——	0.109	(100)	4.40	(100)
	after incubation	0	1.07	−	0.106	(97)	4.67	(106)
				+	0.120	(110)	5.19	(118)
		0.5	0.8	−	0.163	(149)	6.20	(141)
				+	0.198	(182)	6.16	(140)
		2.0	0	−	0.178	(163)	6.21	(141)
				+	0.223	(205)	6.70	(152)
			1.07	−	0.172	(158)	6.21	(141)
				+	0.220	(202)	6.70	(152)

* Values of fresh weights or of carotene contenrs obtained before or after incubation of the disks. Figures in brackets represent relative values, the initial values being taken as 100. In experiment A the disks weighing 0.113 g and containing 4.19 μg of carotene were incubated for fourteen days, and in experiment B the disks weighing 0.109 g and containing 4.40 μg of carotene were incubated for twelve days.

the 2% sucrose medium also showed no effect. Therefore, changes in tonisity of the medium seemed to have no effect on both the amount of carotene synthesized and the growth of the disks. Accordingly, the effect of the concentration of sucrose as shown in Table III seemed to be caused, not by the change in the tonisity, but by the change in the amount of carbon source in the medium. It is interesting that such a low concentration of sucrose as 0.25% present in the medium was sufficient to cause the synthesis of carotene to a considerable extent.

Effect of the addition of yeast extract: Yeast extract has been known to be a stimulant for carotene synthesis in a mold[11], and to be a growth factor for plant tissues[12]. Therefore, addition of yeast extract to the medium was assumed to cause enhanced synthesis of carotene by the carrot disks. However, in contrast to the apparent growth promotion, addition of yeast extract caused no remarkable stimulation of carotene synthesis in the disks (Table IV). Since the addition of yeast extract to the medium had no deleterious effect, it was added to the medium in a concentration of 0.1% to maintain the best physiological condition of the carrot disks.

Addition of possible precursors to the medium: β-Methylcrotonic acid and β-hydroxy-β-methylglutaric acid have been known to be the intermediates in the biosynthesis of cholesterol from acetate[13]. Similarly, it has been known that carbon atoms of acetate[14] and of leucine[2,15] were incorporated into the carotene molecule

11) J. Friend, T.W. Goodwin and L.A. Griffiths, *Biochem. J.*, **60**, 649 (1955).

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13) F. Lynen, *Klinische Wochenschrift*, **35**, 213 (1957).

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Table V. Effect of Addition of Acetic Acid to the Medium on the Formation of Carotene in the Carrot Disks.

	concentrations of sucrose	concentrations of acetic acid	fresh weights of the disks*		carotene contents in the disks*	
before incubation	——	——	0.123 g	(100)	4.76 μg	(100)
after incubation	0 %	0 %	0.143	(116)	5.36	(113)
		0.01	0.149	(121)	5.38	(113)
		0.1	0.154	(125)	5.32	(112)
	0.25	0	0.178	(145)	5.87	(124)
		0.01	0.183	(149)	5.52	(116)
		0.1	0.161	(131)	5.10	(107)
	2	0	0.251	(205)	6.27	(132)
		0.01	0.250	(203)	7.00	(147)
		0.1	0.187	(152)	5.28	(111)

* Values of fresh weights or of carotene contents obtained before or after incubation of the disks weighing 0.123 g and containing 4.76 μg of carotene for thirteen days. Figures in brackets repesent relative values, the initial values being taken as 100.

Table VI. Inhibition of Carotene Synthesis in the Carrot Disks by Lactic Acid and β-Methylcrotonic Acid

	added substances	concentrations of added substances	fresh weights of the disks*		carotene contents in the disks*	
before incubation	——	——	0.122 g	(100)	6.26 μg	(100)
after incubation	none	——	0.304	(249)	9.19	(147)
	β-methylcrotonic acid	0.1 %	0.161	(132)	6.49	(104)
		0.01	0.309	(253)	8.97	(143)
	dl-lactic acid	0.3	0.179	(147)	6.60	(105)
		0.03	0.321	(263)	9.89	(158)

* Values of fresh weights or of carotene contents obtained before or after incubation of the disks weighing 0.122 g and containing 6.26 μg of carotene for thirteen days. Figures in brackets represent relative values, the initial values being taken as 100. Substances were added as free acid and pH of the medium was adjusted to that of the 2% sucrose-medium with sodium carbonate.

synthesized by some microorganisms. Recently, mevalonic acid[13,16] was shown to be a more direct precursor for cholesterol synthesis. Moreover, Goodwin and other investigators[3,17] demonstrated the incorporation of mevalonic acid into the carotene molecule by some organisms including carrot slices. Therefore, it may be expected that additions of these substances to the medium stimulate the synthesis of carotene by the carrot disks. Mevalonic acid, β-methylcrotonic acid, leucine, β-hydroxy-β-methylglutaric acid, acetic acid and lactic acid were tested. Addition of 0.1% acetic acid, 0.1% β-methylcrotonic acid, or 0.3% *dl*-lactic acid (equivalent to 0.1% acetic acid) showed inhibitory effects on both the growth of the disks and the formation of carotene. At lower concentrations equivalent to 0.01% acetic acid, these substances showed neither stimulation nor inhibitory effect on both the growth and the formation of carotene. β-Hydroxy-β-methylglutaric acid and leucine showed no remarkable inhibitory effect on both the growth and the formation of carotene, even when they [were added to the media at concentrations equivalent to 0.1% acetic acid. Neither of them, however, showed a significant

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17) G.B. Balow and A.D. Patrick, *Nature*, **182**, 662 (1958).

TABLE VII. EFFECT OF ADDITION OF MEVALONIC ACID TO THE MEDIUM ON THE FORMATION OF CAROTENE BY THE CARROT DISKS.*

	concentrations of sucrose	concentrations of *dl*-mevalonic acid	fresh weights of the disks**		carotene contents in the disks**	
before incubation	——	——	0.128 g	(100)	6.24 μg	(100)
after incubation	0 %	0 %	0.151	(126)	6.58	(106)
		0.01	0.152	(127)	6.90	(111)
	0.25	0	0.202	(158)	7.00	(112)
		0.01	0.198	(154)	7.13	(114)
	2.0	0	0.337	(263)	9.11	(146)
		0.01	0.308	(240)	8.95	(144)

** Values of fresh weights or of carotene contents obtained before or after incubation of the disks weighing 0.128 g and containing 6.24 μg of carotene for fourteen days. Figures in brackets represent relative values, the initial values being taken as 100.

stimulating effect on both the growth and the synthesis of carotene by the disks, except when β-hydroxy-β-methylglutaric acid was added to the 2% sucrose-medium at the concentration of 0.009%. In the last case, a positive effect of the added acid was observed. Nevertheless, the effect of β-hydroxy-β-methylglutaric acid was not consistently reproducible. Addition of *dl*-mevalonic acid to the medium at concentrations of 0.01～0.026% also showed no effect. Effects of the addition of acetic acid, lactic acid, β-methylcrotonic acid and mevalonic acid are shown in Tables V～VII, and a positive, but not reproducible, effect of β-hydroxy-β-methylglutaric acid is shown later in Table IX.

Inhibition of carotene synthesis by diphenylamine: Diphenylamine has been known to be an inhibitor for the carotene synthesis and a stimulant for the synthesis of colorless polyenes in some microorganisms[2]. Therefore, the effect of the addition of this substance to the medium was investigated. In contrast to the case of microorganisms, at certain concentrations where the inhibition of carotene synthesis was observed (Table VIII), diphenylamine inhibited the growth of the disks. Moreover there was no indication of the stimulation of synthesis of colorless polyenes. The shapes of the absorption spectra of the extract from the normal carrot disks and that from the disks grown on the medium containing diphenylamine were essentially the same (Figure 2). The rise in light absorption in the region 335 mμ could not be attributed to the formation of colorless polyenes.

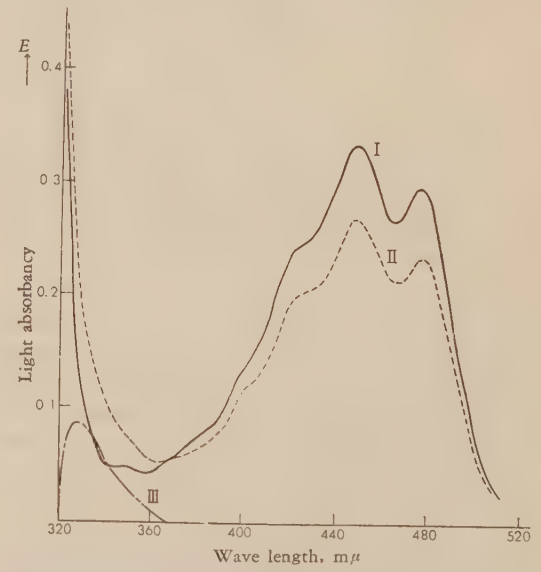

FIG. 2. Absorption Spectra of the Extracts from the Disks inhibited and not inhibited by Diphenylamine.

Curve I: Absorption spectrum of the extract from normal disks.
Curve II: Absorption spectrum of the extract from inhibited disks.
Curve III: Part of the difference spectrum of curve I and II (II-I).

* Another experiment, in which the concentration of mevalonic acid was increased to abour 2.5 times as much, showed similar result.

TABLE VIII. DIPHENYLAMINE INHIBITION OF CAROTENE FORMATION IN THE CARROT DISKS.

	concentrations of diphenylamine	fresh weights of the disks*		carotene contents in the disks*	
before incubation	——	0.160 g	(100)	9.06 μg	(100)
after incubation	0	0.389	(243)	12.5	(138)
	10^{-6} M	0.409	(256)	12.6	(139)
	10^{-5} M	0.350	(219)	12.0	(132)
	1.6×10^{-5} M	0.260	(153)	10.1	(111)

* Values of fresh weights or of carotene contents obtained before or after incubation of the disks weighing 0.160 g and containing 9.06 μg of carotene for twelve days. Figures in brackets represent relative values, the initial values being taken as 100.

TABLE IX. A RESULT INDICATING A STIMULATING EFFECT OF THE ADDITION OF β-HYDROXY-β-METHYLGLUTARIC ACID TO THE MEDIUM ON THE FORMATION OF CAROTENE IN THE CARROT DISKS.

	concentrations of sucrose	concentrations of β-hydroxy-β-methylglutaric acid	fresh weights of the disks*		carotene contents in the disks*	
before incubation	——	——	0.128 g	(100)	7.58 μg	(100)
after incubation	0	0	0.152	(119)	8.05	(106)
		0.009	0.144	(112)	7.95	(105)
	0.25	0	0.221	(172)	10.86	(142)
		0.009	0.201	(157)	10.29	(137)
	2.0	0	0.308	(240)	11.79	(156)
		0.009	0.377	(295)	14.90	(197)

* Values of fresh weights or of carotene contents obtained before or after incubation of the disks weighing 0.128 g and containing 7.58 μg of carotene for fourteen days. Figures in brackets represent relative values, the initial values being taken as 100.

At about 370 mμ, where phytofluene should show maximum absorption, any increase in light absorption was not observed. The same is the case at 430 mμ where ζ-carotene is to exhibit maximum absorption. Although a possible increase in phytoene content could not be ruled out, synthesis of colorless polyenes seemed to be not stimulated significantly by the addition of diphenylamine to the culture medium. Therefore, it is unlikely that diphenylamine is a specific inhibitor for the carotene synthesis by the carrot root.

DISCUSSION

It is apparent from the preceding data that the disks of carrot root can synthesize a considerable amount of carotene during their growth. Although yeast extract stimulated the growth of the disks fairly well, it did not cause an increased synthesis of carotene by the disks. It suggests that the growth of the disks is not necessarily accompanied by the synthesis of carotene. In fact, in some cases, when the disks were prepared from a root after long storage, no synthesis of carotene was observed in spite of the active growth. Moreover, even the disks, which were prepared from the xylem of carrot root exclusive of the cambium layer and showed very poor growth, sometimes synthesize carotene as the disks including the cambium layer did.

Attempts to demonstrate any stimulating effect of possible precursors on the carotene synthesis by the carrot disks were unsuccessful. Nevertheless, participation of these substances to the synthesis of carotene could not be ruled out. A result is obtained indicating a stimulating activity

of β-hydroxy-β-methylglutaric acid, as shown in Table IX, although this stimulating activity was observed only in two cases and not decidedly confirmed. It is interesting that this stimulation was observed only when a sufficient amount of sucrose was present in the medium. Recent progress in studies on the cholesterol biosynthesis shows that cholestered is derived directly from mevalonic acid[18]. The fact that mevalonic acid showed no stimulating effect on the synthesis of carotene by the disks may be attributed to its poor permeability through cell walls of the carrot root, or to the absence of mevalonate-activating system in the carrot cells. Another possibility is that a sufficient amount of a precursor for carotene synthesis may be present in the carrot cells, and sucrose in the medium may merely provide energy for synthesis of carotene from this precursor.

The difference in the modes of diphenylamine inhibition of carotene synthesis in microorganisms and in the carrot disks, as shown in Table VIII and Figure II, may suggest the presence of different synthetic mechanisms working in the carrot and in microorganisms.

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2, p. 211~216, 1960]

Metabolism of Aromatic Compounds by Microbes

Part VI. Existence of Two Pathways, the Pathway *via* Protocatechuic Acid and the One *via* Gentisic Acid, in the Metabolism of *m*-Hydroxybenzoic Acid by Bacteria

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The metabolic products of *m*-hydroxybenzoic acid formed by certain *Pseudomonas*, *Micrococcus*, and *Bacterium* strains which possess oxidizing ability of this acid were detected by paperchromatography. It was recognized that protocatechuic acid or gentisic acid are intermediary metabolites of *m*-hydroxybenzoic acid by these bacteria and the both acids are not detected in one cultural broth.

The authors reported the metabolic pathways of *m*-hydroxybenzoic acid by various bacteria using the technique of sequential induction method and presumed that this acid was metabolized either *via* gentistic acid (2,5-dihydroxybenzoic acid) or *via* protocatechuic acid (3,4,-dihydroxybenzoic acid)[1].

In the previous reports, details in concern of the factors infulencing adaptive formation of the enzymes which oxidatively split *m*-hydroxybenzoic acid (*m*-HBA) and gentisic acid (GA) by *Pseudomonas ovalis* S-5 have been described[2,3]. On the metabolism of *m*-HBA by microbes, N. Walker[4] and B.S. Roof et al.[5] reported on their experiments conducted on specificities and strength of metabolic activities of several bacterial strains on this acid. However, the metabolic pathway of *m*-hydroxybenzoic acid has so far not been elucidated.

In this report detection of the metabolic products of *m*-HBA by the fifteen strains of bacteria which possess oxidizing ability of this acid by paperchromatography is described.

EXPERIMENTS

Biological: Fifteen strains of *m*-HBA metabolizing bacteria which were identified taxonomically in the previous report[6] were used.

Method of culture: Stationary or shaking culture at 30°C were made on synthetic medium containing *m*-HBA as the sole carbon source. Stationary culture was carried out in 1,000 ml of the medium in a 2,500 ml Fernbach flask and the cells were harvested after 4 days. Shaking culture was carried out in 100 ml of the medium in a 500 ml Sakaguchi flask using reciprocal shaker (130~140 r. p. m. 7 cm) and the cells were harvested after 16~18 hours.

Cultural medium.

Nitrogen source: As nitrogen source, three compounds such as $(NH_4)_2SO_4$, NH_4NO_3 and $(NH_4)H_2PO_4$, were tested for the accumulation of gentisic acid and protocatechuic acid and were proved to have no difference.

Influence of pH: Three levels of initial pH value such as 5.2, 7.2 and 8.5 were examined, and as shown

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5) B. S. Roof, T. J. Lannon and J. Turner, *Proc. Soc. Expt. Biol. Med.*, **84**, 38 (1953).
6) K. Arima, K. Komagata, S. Sugiyama and M. Kazama, *J. Agr. Chem. Soc. Japan*, **28**, 635 (1954).

TABLE I. THE EFFECT OF pH ON PRODUCTION OF GENTISIC ACID OR PROTOCATECHUIC ACID BY SEVERAL BACTERIA

	pH value		Detection			
	Start.	After culture	U.V. ray*	$FeCl_3$	Diazo-reaction	Pathway**
Micrococcus ureae S-4	5.2	5.2	−	−	+	G
	7.2	7.2	+	−	±	
	8.5	7.8	+	−	+	
Ps. ovalis S-5	5.2	5.4	−	−	+	G
	7.2	7.2	++	−	+	
	8.5	8.0	++	−	+	
Bact. sp. Ca-1	5.2	5.4	−	−	+	G
	7.2	6.6	±	−	+	
	8.5	7.6	−	−	+	
Ps. cruciviae P-8-2	5.2	5.6	−	−	±	P
	7.2	7.0	−	+	±	
	8.5	7.6	−	±	±	
Ps. dacunhae R-10-2	5.2	5.4	−	−	+	P
	7.2	6.6	−	±	+	
	8.5	7.8	−	−	+	
Ps. dacunhae G1-7	5.2	5.6	−	−	+	P
	7.2	6.6	−	+	+	
	8.5	7.8	−	−	+	

Surface culture at 30°C for 20 hrs was made on synthetic medium (see text).
* Blue fluorescence
** The results of investigation by sequential induction method.
G — Gentisic acid pathway
P — Protocatechuic acid pathway

in Table I, pH 7.2 was proved to be best for the accumulation of GA. The composition of the synthetic medium which will be used in this experiment is as follows;

m-hydroxybenzoic acid	0.5 per cent
$(NH_4)_2SO_4$	0.1 //
$MgSO_4 \cdot 7H_2O$	0.15 //
KH_2PO_4	0.1 //
pH.....7.2	

Preparation of samples for chromatography from cultural broth.

The procedure is as follows;

1,000 ml cultural broth

↓ centrifugation at 3,000 r.p.m. 30 min. concentration in vacuum to 50 ml; acidified by H_2SO_4 to pH 2.0; ether extraction (cold) by a separation funnel, 3 times

Extraction by cold ether (Fraction 1)

Residual aqueous solution

↓ continuous hot ether extraction for 40 hours

Fraction of hot ether extraction (Fraction 2)

Residue

↓

Addition of 2,4-dinitrophenylhydrazine (Fraction 3)

Preparation of samples of chromatography from incubation mixture.

The bacteria were cultured on bouillon-peptone broth which contained 1 per cent *m*-HBA, and was harvested by centrifugation after 18 hours. Ten g of the wet cells were suspended in 100 ml of M/15 phosphate buffer at pH 7.2 with 300 mg GA or PCA, and were incubated at 30°C on the shaker. Sampling was carried out at an interval of each 2 hours, and the production of ketoacids were examined as their 2,4-dinitrophenylhydrazone.

Paperchromatography.

a) Filterpaper: The Tôyô filterpaper No. 50 (2×40 cm). Before development, filterpaper was saturated beforehand by the vapor of developing solvent in order to prevent the tailing of samples.

b) Developing solvents: After a survey was made for the purpose of detection of various intermediates, the following developing solvents were used;

1) Benzene: Acetic acid: Water (2 : 2 : 1)[7]

2) Butanol; Ethanol: $(NH_4)_2CO_3 \cdot NH_4OH$ buffer (4 : 1 : 2)[8,9]

7) H. G. Bray, W. U. Thorpe and K. White, *Biochem. J.*, **46**, 271 (1951).

8) D. Cavalini, N. Frontali and G. Toschi, *Nature*, **163**, 568 (1946).

9) M. E. Fewster and D. H. Hall, *Nature*, **168**, 78 (1951).

3) 3 per cent NH_4OH saturated butanol

4) Butanol: Formic acid: Water (4 : 1.5 : 1)

The solvents 1) and 2) were generally used for development of the aromatic compounds. The solvents 2) and 3) for development of 2,4-dinitrophenylhydrazone of keto acids, and the solvent 4) for fatty acids. Each solvent was applied from the mixing after 3 to 4 days.

c) Development was carried out at 20°C by the ascending method.

d) Detection of compounds: The indicators to detect each compound are follows;

1. *m*-hydroxybenzoic acid: the diazo reaction was used.

The first solution; 1 per cent *p*-aminoacetophenone in 1 N H_2SO_4

The second solution; 1 N HNO_2

After the developed papers were dried at room temperature, the first and then the second solution were sprayed and the reddish orange spots appeared.

2. Gentisic acid: This was detected by the blue fluorescence when the paper was exposed in ultra violet rays in the dark room or by the brown spots by reaction with $FeCl_3$ (1 per cent $FeCl_3$ alcoholic solution was sprayed). Among these, the most convenient and sensitive indicator was found to be its own blue fluorescence.

3. Protocatechuic acid: The detection of the acid was either due to the diazo reaction similar to the case of *m*-HBA, or due to its dark green spot by reaction with $FeCl_3$. Usually, the dark green spot by spraying $FeCl_3$ was used.

4. Other phenolic compounds: These were mainly detected by the reaction with $FeCl_3$, and when the coloration was weak, alkalic solution was used as the indicator. Salicylic acid has a specific fluorescence under the ultra violet rays, and was detected by such characteristics.

5. Keto acids: The detection of keto acid was due to yellow spots of their 2,4-dinitrophenylhydrazone.

6. Aliphatic acids: After development, the paper was put in a drier at 110°C for 10 min. Then bromcresolpurpule solution (0.16 per cent in 5 per cent alcohol adjusted to pH 8.0) was sprayed and yellow spots were revealed on the blue back ground.

RESULTS

R_F values of several related compounds.

R_F values of various phenolic acid and several other acids which were related with the terminal oxidants of these acids were estimated by different solvents. Table II shows the R_F values of these compounds developed by different solvents. These values are the average of several experiments and pyruvate was generously shown to have two spots when developed with the solvent 2.

TABLE II. R_F VALUES OF THE RELATED COMPOUNDS OF MONOHYDROXYBENZOIC ACIDS WITH VARIOUS SOLVENTS

	Solvent I	II	III	IV
o-hydroxybenzoic acid	0.81	0.62		0.95
m-hydroxybenzoic acid	0.55	0.40		
p-hydroxybenzoic acid	0.72	0.34		
2, 3-dihydroxybenzoic acid	0.40	0.38		
β-resorcilic acid	0.27	0.40		
protocatechuic acid	0.12	0.18		0.85
gentisic acid	0.25	0.49		0.88
catechol	0.44	0.89		
resorcine	0.16	0.94		
hydroquinone	0.17	0.87		
2, 4-dinitrophenylhydrazone of levulic acid		0.48		
pyruvic acid		0.41, 0.61	0.34	
α-ketoglutaric acid		0.092	0.06	
oxaloacetic acid		0.14	0.07	
levulic acid				0.76
succinic acid	0.28			0.74
fumaric acid				0.81
maleic acid	0.18			0.47
citric acid	0.22			0.35

Solvent I: Benzene 2: Acetic acid 2: Dist water 1
II; *n*-Butanol 4: Ethanol 1: 1 N $(NH_4)_2CO_3$ buffer 2
III; Butanol 3 per cent NH_3
IV; *n*-Butanol 4: Formic acid 1.5: Dist water 1.

The detectable concentration of related compounds.

As shown in Table III, *m*-HBA was detectalbe from 3 gamma by the Diazo reaction, GA was detectable from 3 gamma by alkalic $FeCl_3$ solution, but from only 1 gamma by its specific blue fluourescence. PCA was detectable from 3 gamma with its green spot by $FeCl_3$ solution. Keto acids were detectable from 1 gamma in the form of their 2,4-dinitrophenylhydrazone. In the detetion of fatty acids it was necessary to use at least 100 gamma of them for bromcresol-purple as an indicator.

Detection of aromatic compounds from stationary cultural broth.

R_F values obtained from the each Fraction 1 of the cultural broth of the several strains developed by the solvent 1 are shown in Table IV. Since the spots, of which the R_F value

TABLE III. THE DETECTABLE CONCENTRATION OF RELATED COMPOUNDS BY SEVERAL REAGENTS ON PAPERCHROMATOGRAPHY

	Concentration in spoting (γ)	Detection			
		$FeCl_3$	$FeCl_3$ $+NH_4OH$	U.V. ray	Diazo-reaction
gentisic acid	5	+	+	+	
	3	−	+	+	
	1	−	−	+	
protocatechuic acid	5	+	+	−	+
	3	+	+	−	+
	1	−	−	−	−
m-hydroxybenzoic acid	5				+
	3				+
	1				−

Developing solvent was the upper layer of a mixture of benzene 2, acetic acid 2, and water 1.

TABLE IV. R_F VALUES OF THE INTERMEDIATES OF *m*-HYDROXYBENZOIC ACID IN FRACTION 1 IN STATIONARY CULTURE

Strain	R_F Value	
Micrococcus ureae S–4	0.52	0.24
Ps. ovalis S–5	0.54	0.25
Bact. sp. Ca–1	0.57	0.27
Bact. sp. A–10–2		0.24
Micrococcus varians P–10–7	0.56	0.24
Ps. dacunhae G1–13	0.53	0.12
Ps. dacunhae G1–7	0.54	0.11
Ps. dacunhae R–10–2	0.55	0.11
Ps. dacunhae A–6–3	0.54	0.10
Ps. dacunhae A–7–2	0.54	0.12
Ps. cruciviae P–8–2	0.52	0.12
m-hydroxybenzoic acid	0.55	
gentisic acid		0.25
protocatechuic acid		0.11

developing solvent was the upper layer of a mixture of benzene 2, acetic acid 2, water 1.
Control were prepared as acetone solution.

was about 0.52 gave a negative reaction with $FeCl_3$ while by the Diazo reaction gave a clear reddish orange, this substance was considered to be *m*-HBA. The spot with a R_F value of 0.24~0.25 was proved to be GA, since their R_F and specific color reaction were the same as the synthetic samples GA was detected from the cultural broth of the strains S-4, S-5, A-10-2, and P-10-7. The strains G1-7, G1-13, R-10-2, A-6-3, A-7-2 and P-8-2 produced spots which were identifical with PCA.

The spot with a R_F value of 0.27 produced by strain Ca-1 was most probablly GA due to its fluorescence and to the results of coloring reations, although the R_F value of 0.27 also corresponded to β-resorcilic acid.

Detection of aromatic compounds from shaking culture broth.

Table V shows the results of detection of the intermediate from shaking cultural broth. As well as in the stationary culture, GA was recognized in the cultural brothes of strains S-4, S-5, while PCA was detectable in those of strains G1-7, R-10-2 and P-8-2.

Summarizing the above results, as seen in Table VI, the same conclusion from the experiment with the sequential induction method was obtained. That is, *m*-HBA was oxidized *via* gentisic acid or protocatechuic acid according to bacterial strain, respectively.

Detection of keto acids.

As described above, the distinction between

TABLE V. R_F VALUES OF THE INTERMEDIATES OF *m*-HYDROXYBENZOIC ACID IN FRACTION 1 IN SHAKING CULTURE

Strain	R_F Value	
Micrococcus ureae S–4	0.51	0.25
Ps. ovalis S–5	0.52	0.24
Ps. dacunhae G1–7	0.57	0.12
Ps. dacunhae R–10–2	0.52	0.11

Developing solvent was the upper layer of a mixture of Benzene 2, acetic acid 2, water 1.

Table VI. Summarizing the Production of Gentisic Acid or Protocatechuic Acid by *m*-Hydroxybenzoic Acid Assimilating Bacteria

Strain	Gentisic acid	Protocatechuic acid
Micrococcus ureae S-4	+	−
Ps. ovalis S-5	+	−
Bact. sp. Ca-1	+	−
Bact. sp. A-10-2	+	−
Micrococcus varians P-10-7	+	−
Ps. dacunhae G1-7	−	+
Ps. dacunhae G1-13	−	+
Ps. dacunhae R-10-2	−	+
Ps. dacunhae A-6-3	−	+
Ps. dacunhae A-7-2	−	+
Ps. cruciviae P-8-2	−	+

Table VII. R_F Values of the Intermediates of *m*-Hydroxybenzoic Acid in Fraction 3 in Shaking Culture

Time (culture)	*Ps. ovalis* S-5		*Ps. dacunhae* G1-7	
	Spot I	Spot II	Spot I	Spot II
2	0.12	0.46		
4	0.16	0.49	0.14	
6	0.13	0.49	0.12	
8	0.13	0.49	0.13	
10	0.14	0.41	0.14	0.41
12	0.17	0.47	0.21	0.50
14	0.16	0.42	0.18	0.46
16	0.16	0.44	0.18	0.48
18	0.17	0.44	0.18	0.48
24	0.16	0.44	0.21	0.44

Developing solvent was the upper layer of a mixture of *n*-butanol 4, ethanol 1, water 2.

the strains having GA pathway and the other having the PCA pathway, was recognized. The detection of keto acids was carried out from incubation mixtures of *Ps. ovalis* S-5 and *Ps. dacunhae* G1-7. The former was oxidized *m*-HBA *via* GA, the latter oxidized *m*-HBA *via* PCA. An amount of 20 ml of samples was taken every 2 hours, and 2,4-dinitrophenylhydrazone was prepared and then developed in solvent 2. As seen in Table VII, different acids were detected depending upon the bacterial strain. In the case of *Ps. ovalis* the composition of acids was constant irrespective of the time of culture. Spot 1 of the former R_F value of 0.12 corresponds to α-ketoglutaric acid but assumed an orangish red color which is quite different from that of 2,4-dinitrophenylhydrazone of α-ketoglutaric acid. In the chromatogram of the mixture with the authentic α-ketoglutaric acid, the keto acid in question gave different spots hence the possibility that this keto acid is α-ketoglutaric acid was excluded. As for the spot 1 of *Ps. dacunhae*, from the R_F value it seemed to be 2,4-dinitrophenylhydrazone of α-ketoglutaric acid from paperchromatography mixture with the authentic samples. In the other case, spot 11 was not detectable between 2 to 8 hours' incubation, and was considered to be 2,4-dinitrophehylhydrazone of pyruvate.

DISCUSSION

These results do not clearly indicate successive steps of the metabolic pathway of *m*-HBA. However, they agreed with the assay of sequential induction method, which concluded that two metapolic pathways exist in the oxidation of *m*-HBA and its pathways are specific by bacterial strain. Namely, the existence of a common precursor is considered in the induced enzyme formation between analogous compounds, but against one inducer the bacterial metabolic activity differs between bacterial strains. R. Y. Stanier and N. Tsuchida[10] in bacterial metabolism of tryptophan, previously demonstrated that there were two pathways, one the aromatic path *via* anthranilic acid, the other the quinoline path *via* quinolic acid, but both pathways did not exist together in one strain.

The production of lower keto acids are intermediary steps to terminal oxidation as well as the metabolism of aromatic compounds investigated by S. Dagley[11] and M. Isono[12]. An unknown keto acid produced by *Ps. ovalis* S-5 is considered to be a direct intermediate cleavaging benzene ring of GA. As will described in the

10) R. Y. Stanier and M. Tsuchida, *Biochem. J.*, **58**, 45 (1949).
11) S. Dagley, M. E. Fewster and F. C. Happold, *Biochem. J.*, **63**, 327 (1952).
12) M. Isono, *J. Agr. Chem. Soc. Japan*, **27**, 260 (1953).

latter report[13], the present authors assumed 7 carbons diketo-dicarboxylic acid, probably fumarylpyruvic acid, from the results obtained by manometrical experiment of gentisic acid oxidase. Therefore, considering the spot of its hydrazone not having appeared in developing Fraction 3 from cultural broth, it seems that metabolic decomposition of it had already been carried out or decomposition during prolonged hot ether extraction and other treatment had occurred.

SUMMARY

Oxidation of *m*-HBA by the eleven bacterial strains which were able to metabolize this compound as a sole source of carbon was carried out. The cultural broths were examined by paperchromatography for detection of intermediates.

Gentisic acid was identified in the culture filtrate of the five strains, while protocatechuic acid by the remaining six strains. Protocatechuic acid was not detected in all the cultural broth of bacteria which belong to the gentisic acid path type, and *vice versa*.

Strains which possess the gentisic acid path were *Pseudomonas ovalis* (two strains), *Micrococcus ureae*, and an unidentified *Bacterium* and a strain of *Aerobacter*. Strains which have the protocatechuic acid path were *Pseudomonas dacunhae* (five strains) and *Pseudomonas cruciviae*.

An unknown keto acid was detected as its 2,4-dinitrophenylhydrazone from the incubation mixture of the resting cell of *Pseudomonas ovalis* S-5 and gentisic acid. This new keto acid was most probably the direct intermediate after the cleavage of gentisic acid.

Acknowledgement The authors wish to express their sincere thanks to Prof. Emeritus K. Sakaguchi for his continous guidance throughout this work.

13) S. Sugiyama, K. Yano, H. Tanaka and K. Arima, This Bulletin, in press.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2, p. 217~218, 1960]

Analysis of Isomeric Menthols by Gas-Chromatography

Sir:

One of the basic problems in the study on the synthetic menthol is to establish an effective method for the analysis of a mixture of isomeric menthols. Any reliable method, however, has not been known to estimate the quantity of isomer in a menthol mixture. Recently gas-chromatography of peppermint oil has been carried out by using high vacuum oil[1] and silicone oil column[2]. In the latter case, (−)-menthol and (+)-neomenthol were detected, but a considerable overlap between the two isomers was observed in the chromatogram. We have found that such overlap is not observed on polyethylene glycol column. Moreover, the other geometrical isomers, isomenthol and neoisomenthol, are also separated on this column.

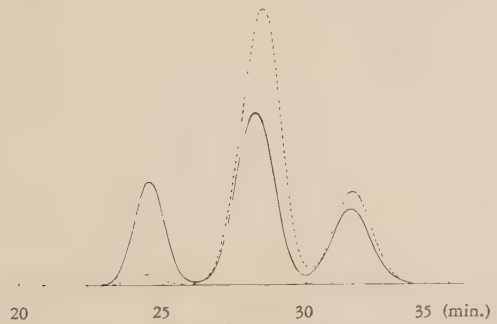

FIG. 1. Two Chromatograms

——, Mixture of four isomeric menthols.
------, Product obtained by the reduction of (−)-isomenthone with sodium.

Gas: He, Column (0.6 cm×3 m): polyethylene glycol (6000), Column Temp.: 160°C, Flow Rate: 59.0 ml/min., Apparatus: GC-1A (Shimadzu).

The chromatogram of the mixture of equal amounts of four isomeric menthols shows three elution peaks (Fig. 1). The retention time of the first and the third peak (R_{t_2}, Table I) is the same with that of (+)-neomenthol (I) and (+)-isomenthol (IV) (R_{t_1}, Table I) respectively. In this case, IV instantly crystallizes when it emerges from the column. On the other hand, the retention time of the second peak (R_{t_2}, Table I) is just in the middle between individual retention time of (−)-neoisomenthol (II) and (−)-menthol (III) (R_{t_1}, Table I). This second peak corresponds to the co-elution of II and III, and moves toward that of either of II or III when one of them is removed from the mixture.

TABLE I. RETENTION TIME* OF ISOMERIC MENTHOL

	b.p.** (°C)	R_{t_1} (min.)	R_{t_2} (min.)
(+)-neomenthol (I)	212	24.5	24.5
(−)-neoisomenthol (II)	215	27.8	28.2
(−)-menthol (III)	216	28.5	
(+)-isomenthol (IV)	219	31.5	31.5

*): The time required during the sample injection and the appearance of elution peak; Rt_1 is obtained from the individual chromatogram of the isomers, while Rt_2 is obtained from the chromatogram of the mixture of isomers (Fig. 1).
**): From Simonsen's "The Terpenes," Cambridge University Press, 1953, Vol. I. p. 243.

The above failure in the separation of II and III, however, is not serious objection to the practical purpose, since co-production of II and III by any reaction is rare case, and even if it occurs, II is easily detected by infrared spectrum[3,4]. As a typical example of the present analytical method, the chromatogram of a menthol mixture obtained by the reduction of (−)-isomenthone with sodium in anhydrous ethanol is shown in Fig. 1. This figure indicates that neomenthol (I) is formed (2%) by this reduction (first peak). Such detection and

1) Presented at the 12 anual meeting of Chemical Society of Japan, April 4, 1959, by H. Kawahara and K. Abe.
2) A. Komatsu, *Koryo*, **55**, 30 (1959).
3) H. Ueda and Tetsuo Mitsui, *J. Agr. Chem. Soc. Japan*, **28**, 945 (1954).
4) Y. R. Naves and J. Lecomte, *Bull. Soc. Chim. France*, **1955**, 792.

estimation of the trace component is not possible by any hitherto known method.

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Synthesen und Konfigurationsermittlung in der Rotenoid-Reihe

XIII[1] Synthese der Tubasäure

2-(α-Hydroxy-isopropyl)-4-benzyloxy-cumaron (I)[2,3] geht durch katalytische Hydrierung in Gegenwart von Raney-Nickel unter Aufnahme von 2 Mol. Wasserstoff glatt und zwar in ausgezeichneter Ausbeute in *dl*-Hydroxydihydrotubanol (IIa) über. Schmp. 150~151°. $C_{11}H_{14}O_3$(194.2) Ber. C 68.02 H 7.27, Gef. C 68.01 H 7.32. Bei der Acetylierung mit Acetanhydrid in Pyridin liefert IIa *dl*-2-(α-Hydroxyisopropyl)-4-acetoxy-cumaran (IIb). Sdp.$_8$ 166~168° n_D^{20} 1.5233 $C_{13}H_{16}O_4$(236.3) Ber. C 66.08 H 6.83, Gef. C 66.08 H 6.93. Durch Behandlung mit Phosphortribromid oder mit Methansulfonylchlorid in Pyridin-lösung geht IIb unter Abspaltung von 1 Mol. Wasser in *dl*-Tubanolacetat (IIIb) über, das aber von dem bei der Dehydratisierung von IIb gleichzeitig sich bildenden Isomeren, dem Roteol-acetat (IVb), nicht abgetrennt werden konnte. Sdp.$_6$ 125° n_D^{11} 1.5335. Das unreine IIIb wurde ohne weitere Anreicherung zum *dl*-Tubanol (IIIa) verseift, das analytisch rein ist aber nach seinem IR-Spektrum etwa 20% Roteol (IVa) enthält. Sdp.$_{5\sim6}$ 134° n_D^{15} 1.5630 $C_{11}H_{12}O_2$(176.2) Ber. C 74.94 H 6.86, Gef. C 74.64 H 7.05 Das Natrium-salz des synthetischen *dl*-Tubanol wurde in Äthylenglykol gelöst und unter Einführung von Kohlendioxydström einigen Stunden auf 180~190° erhitzt. Nach der gewöhnlichen Aufarbeitung wurden eine Carbonsäure vom Schmp. 187~188° erhalten. $C_{12}H_{12}O_4$(220.2) Ber C 65.44 H 5.49 Gef. C 65.60 H 5.46.

IR-Spektrum der letzten Säure in Chloroformlösung stimmt mit demjenigen der linksdrehenden Tubasäure[4~6] von natürlicher Herkunft völlig überein. Daher handelt es sich um *dl*-Tubasäure. Aus der synthetischen Säure wurde l-Säure als das in Äthanol sehr schwer lösliche Brucin-salz rein abgetrennt. Schmp. 231~233° (zers.) Misch-schmp. mit der authentischen Probe (Schmp. 233~235°, zers.) weist keine Erniedrigung auf. Beide IR-Spektren in Nujol-Paste stimmen ganz überein. $[\alpha]_D^{15}$ −8° (c=0.0105, Chloroform) (authentische Probe $[\alpha]_D^{15}$ −12° (c=0.0115, Chloroform)) $C_{35}H_{38}O_8N_2$ (614.7) Ber. C 68.39 H 6.23 N 4.56, Gef. C 67.98 H 6.21 N 4.45

Eine Synthese der *dl*-Tubasäure aus 2-Acetyl-

1) XII. Mitteil., M. Miyano und M. Matsui, *Chem. Ber.*, **93**, im Gedrück (1960).
2) M. Miyano und M. Matsui, Dieses Bulletin, **23**, 141 (1959).
3) M. Miyano und M. Matsui, *Chem. Ber.*, **92**, 2487 (1959).
4) S. Takei, *Biochem. Z.*, **157**, 1 (1925).
5) S. Takei und M. Koide, *Ber.*, **62**, 3030 (1929).
6) T. Kariyone, Y. Kimura und K. Kondô, *Yakugakuzasshi*, **44**, 1049 (1924).
7) T. Kariyone und K. Kondô, *eben da*, **45**, 376 (1925).
8) A. A. Shamshurin, *Zhur. Obshei Khim.*, **21**, 2068 (1951); *Chem. Abstr.*, **46**, 6638 (1952).

4-hydroxy-5-carbomethoxy-cumaron wurde von Shamshur in[8] in 1951 mitgeteilt. Die genannte Synthese bewährt sich entgegen den Behaupten von Shamshurin nicht[1].

Wir werden über diese Arbeit an anderer Stelle ausführlich berichten.

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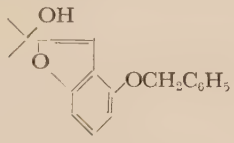
Eingegangen am 25, Januar 1960



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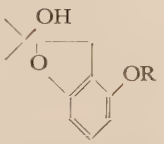
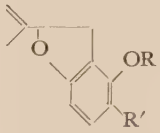
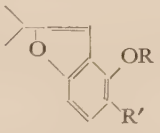


IIa R : H
b R :-$COCH_3$



IIIa R, R′ : H
b R :-$COCH_3$, R′ : H
c R : H, R′ :-CO_2H



IVa R, R′ : H
b R :-$COCH_3$, R′ : H

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 34, No. 1 (1960)

Studies on the Substance produced by *Aspergillus oryzae*. Part I. Fractionation of Amylase and Proteinase in the Enzymes of *Aspergillus oryzae*. (p. *1*~*5*)

By Teruzo SAWASAKI
(*The Institute of Physical and Chemical Research*)

The many enzymes and many activities of these were contained in a culture fluid of *Aspergillus oryzae*. The amylases were separated into two components, Taka-amylase A and B. The former has the starch liquefying power and saccharogenic activity and the latter has the specific precipitation with rivanol or Na_2CO_3-rivanol. Proteinases were also studied extensively in this country. The mold contains at least three kinds of proteinase. Each one acts in alkaline, neutral and rather strong acidic medium.

This report shows the fractionated amylases and proteinase by elution analysis on columns of ion exchanging resins and almina. In each case, the granular aliphatic amine resin (Duolite A 2) or granules acrylic type (Duolite CS 101) and acidic treatment almina had been used. These columns had to be bufferized and eluting solutions also had to be.

The starch liquefying amylase and acidic proteinase were adsorbed in bufferized Duolite A 2, but the saccharogenic amylase was not, which was adsorbed in resin Duolite A 2 of acetate form.

The alkaline proteinase was adsorbed in bufferized Duolite CS 101. All of adsorbed enzymes were eluted with neutral phosphate buffer solution and were fractionated with almina columns.

The chemical character of these enzymes were investigated.

Studies on the Substance produced by *Aspergillus oryzae*. Part II. Precipitant and Isolation of the Starch Liquefying Amylases produced by *Aspergillus oryzae*. (p. *5*~*8*)

By Teruzo SAWASAKI
(*The Institute of Physical and Chemical Research*)

It has been known for several years that starch liquefying amylase was obtained as precipitation with rivanol from "Taka-amylase A" (Akabori et al.).

This report shows the new distribution by eluting analysis on column of ion exchange resins and acidic treatment alminal column. In each case, the starch liquefying amylase, acidic proteinase and saccharogenic amylase were adsorbed in the prepared resin "Duolite A 2" column at acetate form. The enzymes were eluted with 0.2 N sodium phosphate buffer solution at pH 7.0 and the acidic proteinase was adsorbed on Japanese acid clay, was filtered. This filtrate contained the starch liquefying amylase and the saccharogenic amylase. The starch liquefying amylase was adsorbed in the resin "Duolite A 2" that was equilibrated with 0.2 M acetate buffer solution at pH 5.0 and eluted with sodium phosphate buffer at pH 7.0, dialysed with distilled water at 6.0°C to remove salts, again adsorbed into the acidic treatment alminal column. Then, it was eluted with 0.5 M acetate buffer solution at pH 5.0 and 0.2 N sodium phosphate buffer solution at pH 7.0. Thus, two fractions of amylase were obtained and difference of them are optical density at 278 millimicron on the ultraviolet adsorption spectrum, and they were compared with the crystal of "Taka-amylase A".

The eluted solution with sodium phosphate buffer solution at pH 7.0 has been studied with precipitation method from benzol and naphthoic derivatives. The diphenylglycolic acid and O-carboxyl-phenylglycine were applicable to precipitator for the starch liquefying amylase.

Studies on the Decomposition of L-Ascorbic Acid. Part XIII. Browning Reaction of L-Ascorbic Acid. (3) (p. *8*~*12*)

By Shintaro KAMIYA
(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University, Iwata*)

As previously reported, reductone B, one of three reductones found as decomposing products of dehydroascorbic acid by 5% sulphuric acid solution, was identified with 2,3,4-trihydroxypentenic acid-3. On the other hand, as the decomposing products of dehydroascorbic acid in some organic acid (succinic acid, malic acid, citric acid, tartaric acid, and oxalic acid) solutions, reductone A, B, C, furanecarboxylic acid and xylosone were found and brown color of solutions were deeply referred to the formation and decomposition of

reductones. The same results were obtained in the case of decomposition of dehydroascorbic acid in weak acidic (pH 2~6) and neutral solutions except that reductone A and furanecarboxylic acid were not found as decomposing products, while in weak alkaline solution (pH 8~10) only a small quantity of reductone C, L-threonic acid and oxalic acid were found as decomposing products. Dehydroascorbic acid was added in mandarine orange juice and after three weeks the juice turned to reddish brown in color and reductone B, C, L-xylosone etc. were found as decomposing products.

Studies on the Decomposition of L-Ascorbic Acid. Part XIV. Browning Reaction of L-Ascorbic Acid. (4) (p. *13~16*)

By Shintaro KAMIYA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University, Iwata*)

In order to study the browning reaction of L-ascorbic acid in alkali solution, L-ascorbic acid was decomposed by alkali under several conditions, and some organic acids, i.e. L-threonic acid, oxalic acid, glyceric acid, glycolic acid, lactic acid and formic acid etc. and some reductones were obtained as the decomposing products. As the results, the browning reaction of L-ascorbic acid would be chiefly due to the formation of some unstable carbonyl compounds, which were presumably formed as intermediates on the pathway of dehydroascorbic acid decomposition to L-threonic acid, and could be easily converted in browning-color substances in alkali solution.

On the Stereospecific Permeability of Lactic Acid Bacteria to D- and L-Lactic Acids. (p. *16~22*)

By Akira ŌBAYASHI, Sadao IWANO, Iwao KUSAKA and Kakuo KITAHARA

(*Institute of Applied Microbiology, University of Tokyo*)

1) The activities of D- and L-lactic dehydrogenases were measured by the decolorization of 2,4-dichlorophenol indophenol. The activities of these lactic dehydrogenases of intact cells of lactic acid bacteria differ much from those of these sonic lysate. For example, in *L. delbrückii* (D-lactic acid former) activity of L-enzyme is markedly increased by lysis, but D-enzyme shows scarcely any change. And on the contrary, in L-lactic acid former (*L. thermophilus* or *L. bulgaricus*) D-enzyme is activated.

2) By the addition of cetyl trimethyl ammonium bromide (CTAB) or cetyl pyridinium chloride, known as surface activators which have an effect on the permeability of intact bacterial cells, or by treatment with toluol, similar changes in the enzyme activity is obtained. And under the electron microscope, the swelling of the cell surface in these treated cells are quite distinct. From these facts it was suggested that the intact cells should have stereospecific permeability to lactic acid, and discussed the permeability of the cell in relation to the incubation time as well as to the optical property of the lactic acid.

3) Investigating the mode of action of CTAB, it was supposed that CTAB bind tightly with the cells, changing the character of cell surface.

4) Unlike with the β-galactoside permease of *E. coli* reported by Monod, the transport of lactic acid was not inhibited by azide, fluoride, monojodoacetic acid and 2,4-dinitrophenol.

Studies on the Basic Amino Acids of the Soy Sauces and the Seasoning Liquids. Part I. L-Lysine Determination by L-Lysine Decarboxylase. (p. *22~27*)

By Tetsuo HINO, Katsumi ITO, Koichi HAYASHI and Nobuyuki ABE

(*Ajinomoto Co. Ltd.*)

The authors studied the characteristics of L-lysine decarboxylase yielded in *Bacterium cadaveris*, for determining L-lysine content in soy sauces and the seasoning liquids. (Aji-eki etc.).

They acknowledged that 10~12 mg of the enzyme preparation per one flask was to be used very effectively according to the enzymic activity. The enzyme action was always confirmed to be increased by pyridoxal phosphate.

Action treated enzyme preparation was only active for L-lysine, L-hydroxy lysine, and not for any other naturally occuring amino acids. When the enzyme was prepared, acetone treatment gave definite influence to enzymic activity.

L-lysine content in Aji-eki was about 3 times in comparison with that of traditional soy sauces, namely 1.18 g/dl as free lysine in Aji-eki and 0.34 to 0.38 g/dl as total lysine in soy sauces, but the content increased to 0.41 to 0.49 g/dl in hydrolyzed soy sauces.

The difference may be explained partly owing to the presence of non-available lysine which was investigated by DNP-amino acid method, in addition to peptide.

Amino Acid Accumulation and Bacteria. (p. *27~32*)

By Hiroshi IIZUKA and KAZUO KOMAGATA

(*Institute of Applied Microbiology, University of Tokyo*)

From the taxonomical viewpoint, accumulation of amino acids was studied on 20 genera, 72 species, and

334 strains of aerobic bacteria of type cultures. L-Alanine, L-glutamic acid, and L-valine were most frequently produced from glucose or α-ketoglutarate. Amounts or kinds of amino acids accumulated by bacteria were variable by each strain, cultural conditions i.e., stationary or shaking, or composition of media. Generally, facultative anaerobes including *Escherichia*, *Aerobacter*, *Bacillus* etc. were more powerful accumulators than strict aerobes such as *Pseudomonas*. The distinct relationship between bacterial genera or species and accumulation of amino acids was not recognized. However, as an exception, *Pseudomonas lacunogenes* accumulated considerable amount of L-glutamic acid from α-ketoglutarate which was assumed as species pattern. It was concluded that accumulation of amino acid was recognized in many bacteria, but this character was of little value for bacterial taxonomy because powerful accumulators were limited to special strains, and activity was easily modified by cultural conditions.

Studies on the Cell Wall Lytic Enzymes produced by *Streptomyces* Species. Part I. The Strains and their Lytic Activity towards *Saccharomyces*. (p. *33*~*38*)

By Akira FURUYA and Yonosuke IKEDA

(*The Institute of Applied Microbiology, University of Tokyo*)

In order to obtain microorganisms which could attack cell walls of *Saccharomyces*, samples collected from the field were spread on an agar medium containing heat treated baker's yeast. In the first screening, the colonies with clear zone around them on the medium were picked up, and, in the second test, the strains which could produce a lytic factor even by the submerged culture were selected.

Because strain 62, a *Streptomyces albus*, seemed to be mostly suitable for the present purpose among the eight *Streptomyces* and nine bacteria selected, the lytic factor produced by it was studied in detail. It was found that the water soluble fraction after treatment with ammonium sulfate could lyse even the living cells of *Saccharomyces* in contrast to the original broth which did not. Throughout this work, the lytic activity was measured by the turbidimetric method.

Studies on the Cell Wall Lytic Enzymes produced by *Streptomyces* Species. Part II. The Lytic Enzymes of *Streptomyces* No. 62 Strain. (p. *38*~*44*)

By Akira FURUYA and Yonosuke IKEDA

(*The Institute of Applied Microbiology, University of Tokyo*)

The lytic factor produced by *Streptomyces* No. 62 strain was considered to be an enzyme or a mixture of enzymes because it did lyse not only living cells but also the heat treated cells of *Saccharomyces cervisiae*. It lysed isolated cell walls in 30 minutes and lost its activity upon heating at 60°C for 5 minutes. Mereover, when living cells were treated with the enzyme(s), most of them turned into the so-called protoplasts in a short time.

Besides 17 kinds of yeasts including *Hansenula anomala* and *Candida utilis*, some fungi such as *Aspergillus oryzae*, *Penicillium chrysogenum*, and *Neurospora crassa* were lysed by the enzyme(s). As to bacteria, only *Streptococcus* strain S-8 and *Micrococcus lysodeikticus* were found to be sensitive.

It appears that the enzyme(s) is a mixture of a few enzymes since it has a strong protease activity, and, when the protease activity is arrested by an inhibitor, the enzyme(s) lyses yeast cells to a lesser extent.

Preparation of L-Aspartic Acid by Bacterial Aspartase. (p. *44*~*48*)

By Kakuo KITAHARA, Sakuzo FUKUI and Masanaru MISAWA

(*The Institute of Applied Microbiology, University of Tokyo*)

The authors have made an attempt to synthesize L-aspartic acid by enzymic process using bacterial aspartase. As a result of preliminary experiment, *Escherichia coli* No. 2, *Serratia marcescens* and *Bacterium succinicum* were found to be suitable strains for this purpose. A representative experiment for practical applications is as follows: one gram of dried cells of *E. coli* No. 2, enzyme material, were added to the heterogenous substrate medium containing 50 g ammonium fumerate (as free acid basis) in 100 ml, and the reaction was carried out with gentle stirring at 37°C and pH 7.2~7.4. After 3 days all the substrate disappeared and was converted into L-aspartic acid in almost theoretical yield. Most part of the product was crystallized immediately after the adjustment of pH to 2.77, isoelectric point of L-aspartic acid, and could readily be separated from the medium by filtration. Total yield 56 g (99.3 % in theoretical); crystals 51.2 g.

It may be convenient if the intact cells or the growing culture itself were applicable in place of dried cells. In these cases, howeuer, small amount of suitable surface detergent such as cetyl pyridium chloride was shown to be effective for the rapid bioconversion.

Existence of a New Enzyme "β-1,3′-Xylanase" (p. *48*~*53*)

By Sakuzo FUKUI, Takeo SUZUKI, Kakuo KITAHARA and Tomoo MIWA

(*Institute of Applied Microbilogy, University of Tokyo and Faculty of Science, Tokyo University of Education*)

In this paper, the existence of a new enzyme β-1,3′-xylanase was demonstrated. From a shaking cultural broth of *Chaetomium globosum* A 2, purified β-1,3′-xylan hydrolyzing enzyme was prepared and its properties were examined. As a result of comparative hydrolyzing action upon β-1,3′-xylan and five β-1,3′-xylo-oligosaccharides such as xylohexaose, xylopentaose, xylotetraose, xylotriose and xylobiose, it was found that this hydrolyzing enzyme attacks β-1,3′-xylopyranosidic linkage from a terminal bond in substrate molecule and liberates monosaccharide exclusively and should be classified into exo-type polyase. From above findings, this β-1,3′-xylan hydrolyzing enzyme, being free from amylases, cellulase and β-1,4′-xylanases, may be designated by the name *p*-1,3′-xylanase hereafter.

Polarographic Studies on Storage of Meats. Part III. Relation between Protein Waves of Beef and Changes of its Components. (1) (p. *53～58*)

By Tetsujiro OBARA and Yasokichi OGASAWARA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

It has been shown in a previous report that the polarographic wave of each protein from six parts of beef varies in its shape and height with the increase in storage time. But it is not obvious yet what the variation in the protein wave causes.

We have carried out on experiment on the relation between protein waves of the stored beef and changes of its components.

In this experiment, we have examined changes in the Walkiewicz reaction, the pH, the Cu-Folin coloring and the protein wave of the extract from the beef stored at 15°C.

The results of the experiment mentioned above are as follows:

(1) The variations in the Walkiewicz reaction, the pH, the Cu-Folin coloring and the protein wave of each extract from the following six parts of the stored beef—round, plate ribs, short loin, chuck rib poast, and tender loin—are particular to respective extract.

(2) In the case of each extract from six parts of the stored beef, the respective variation in, the Walkiewicz reaction, the pH, the Cu-Folin coloring and the protein wave of an extract is closely related one another.

(3) In the case of water extracts of beef, there is an increase in, the pH value, the rate of the Cu-Folin coloring and the height of the protein wave with the increase in storage time. On the contrary, in the case of 7% NaCl extracts, there is a decrease in the rate of the Cu-Folin coloring and the height of the protein wave with storage time.

Polarographic Studies on Storage of Meats. Part IV. Influence of Amino Acid Concentration on Protein Wave of Beef. (p. *59～66*)

By Tetsujiro OBARA and Yasokichi OGASAWARA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

We are doing the experiment on the relation between polarographic waves from the protein of the stored beef and changes of its components. Particularly, it is desired to clear the relation between protein waves of the stored beef and amino acids which are free in that. To study this problem it is necessary to examine changes of the protein wave obtained by adding indicated amounts of each amino acid to the extract of the beef. For this purpose, we have examined the influence of each amino acid concentration on the protein wave from the extract.

The results of the experiment mentioned above are as follows:

(1) Cysteine, histidine, arginine, tryptophan, aspartic acid, hydroxyproline, and proline, of the amino acids examined by us, greatly affect the protein wave from the extract of the beef.

(2) When aspartic acid, hydroxyproline, and proline are added to the extract of the beef, the double wave of the protein may be transformed into a single one.

(3) There is the remarkable increase in the height of the protein wave from the extract with the increase in the concentration of cysteine and hystidine. On the contrary, there is the decrease in the wave height with the increase in the concentration of ariginine and tryptophan.

(4) The maximum wave of cobalt is completely eliminated by the addition of the high concentrations, among the concentrations examined by us, of hystidine, arginine, aspartic acid, hydroxyproline, and lysine.

Studies on L-Malic Acid Fermentation. Part II. Cultural Conditions for L-Malic Acid Production. (p. *66～70*)

By Shigeo ABE, Takeshi SAITO and Ken-ichiro TAKAYAMA

(*Tokyo Research Laboratory, Kyowa Fermentation Ind. Co., Ltd.*)

Is has been reported by the authors in the previous paper that some strains of *Aspergillus parasiticus*, *Asp. flavus* and *Asp. oryzae* produce L-malic acid in a liquid culture.

In this paper, cultural conditions for L-malic acid production were studied. The results of the experiments are summarized as follows:

1) Peptone, sodium nitrate, urea, ammonium sulfate are the most suitable among the nitrogen sources tested.

2) The addition of $CaCO_3$ is necessary for the acid production and the optimum amount is 6 to 8% by volume of the culture media.

3) Mn^{++}, Al^{+++}, Cr^{++++} promote the accumulation of the acid.

4) No decrease in the production of the acid is provoked by the inhibitors for TCA cycle reaction; at least a part of the acid may be produced by means of CO_2 fixation besides TCA cycle.

Studies on Amino Acid Fermentation. Part II. On the Mechanism of L-Glutamic Acid Fermentation. (I) (p. *70*~*77*)

By Kō AIDA, Kunio OISHI and Toshinobu ASAI

(*The Institute of Applied Microbiology, University of Tokyo*)

Studies were made on the L-glutamic acid fermentation by *B. megaterium* No. 2. Both free and combined amino acids were analysed at the various phases of the fermentation. L-Glutamic acid accumulated in the medium with a small amount of L-alanine and L-leucine. About five per cent of the total cell nitrogen were free amino acids. The most of them was L-glutamic acid and there were also a small amount of L-alanine and L-leucine. The combined amino acid composition of the cell was almost the same throughout the fermentation, with the exception of L-glutamic acid and L-leucine, which increased with the progress of fermentation. The activities of L-glutamic dehydrogenase and transaminase were compared with various strains of *B. megaterium*. Although activities of transaminase were almost the same, activity of L-glutamic dehydrogenase was found to be considerably strong with the strain which was able to produce a large amount of L-glutamic acid.

Studies on Amino Acid Fermentation. Part V. On the Mechanism of L-Glutamic Acid Fermentation. (2) (p. *77*~*83*)

By Kō AIDA, Kunio OISHI, Katsumi SIMIZU and Toshinobu ASAI

(*The Institute of Applied Microbiology, University of Tokyo*)

The oxidation of TCA cycle intermediates by *B. megaterium* No. 2 was very slow, and especially, α-ketoglutarate was hardly oxidized by the cells obtained after 11 hours' incubation, during which time active accumulation of L-glutamic acid was observed. By omitting calcium carbonate from fermentation medium or addition of sodium bicarbonate to it, the formation of L-glutamic acid was decreased and that of L-alanine was increased. The fixation of carbon dioxide during L-glutamic acid fermentation was proved by using $^{14}CO_2$. Radioisotopic activities were found in L-glutamic, α-ketoglutaric and and succinic acids. Seventy six per cent of total radioisotopic activity of L-glutamic acid was found in the α-carboxyl carbon. The activities of oxalacetic decarboxylase and malic enzyme were proved as the enzymes concerning the fixation of CO_2. L-Glutamic dehydrogenase activities of *K. citrophila*, which produces α-ketoglutaric acid from glucose, and *B. megaterium* No. 2 were compared and it was foumed that the activity of the latter is much stronger than that of the former.

Studies on the Requirement of Amino Acids and Vitamins in Yeast. Part VIII. The Effect of Organic Acids on the Growth of *Saccharomyces sake*. (p. *83*~*87*)

By Masahiro TAKAHASHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Several organic acids were examined for their growth inhibitory action in $(NH_4)_2SO_4$-glucose-salts medium.

Glycerophosphate and malonic, propionic and pyruvic acids (10^{-5} M) had no effect on the growth. Succinic and malic acids (10^{-5} M) had weak inhibitory action. Malonic (10^{-4}), propionic (10^{-4}) fumaric (10^{-5}), oxalic (10^{-5}) succinic (10^{-4}) and malic acids (10^{-4}) had strong inhibitory action.

The author has studied the influence of organic acids above described on the growth in the casein hydrolysate glucose-salts medium, in which the yeast can not grow without pantothenic acid of β-alanine.

No growth war observed in the addition of pyruvic, malic, fumaric, succinic and oqalic acid. However, the yeast could grow in the addition of propionic acid, malonic acid or glycerophosphate.

Rheological Studies on Potato Starch. Part IV. Effect of Far-ultra-violet Radiation on Potato Starch. (p. *88*~*94*)

By Shizue TAKAHASHI and Yoshijiro KIHARA

(*Laboratory of Food Chemistry, Faculty of Home Economics, Ochanomizu University, Tokyo*)

When the potato starch was irradiated with radiation of wavelength 2537Å for long hours, its rheological properties considerably changed. These changes were examined by both the static visco-elasticity measurment and the Brabender amylograph. Iron content of the starch was also determined.

On the starch irradiated in air for 15 and 30 hours, temperature at the first change on amylograms curve and viscosity of cold paste rised, the difference between the maximum and minimum viscosity decreased, and shear modulus of rigidity(G) and viscosity coefficient(η) increased. But, on the irradiation in air for 45 and 60 hours, viscosity of paste lowered and shear modulus of rigidity and viscosity coefficient decreased.

The starch treated with dilute ferric chloride solution was more effected under the same condition.

The changes above mentioned were not only due to ozone oxidation, which is secondary produced in the surrounding atmosphere by irradiation.

Since these rheological changes occurring during irradiation of the starch in oxygen was like that in air, it is supposed that the starch might be photochemically oxidized by irradiation in oxygen-containing atmosphere.

Experiments on the starch irradiated in dry nitrogen, carbon dioxide and vacuum will be attemped to confirm whether there are photolysis or not.

On the Isolation Procedure of Ribonucleic Acid from Rice Plant. (p. *95～100*)

By Hiromu YOSHII and Akira KISO

(*Laboratory of Phytopathology, Faculty of Agriculture, Ehime University, Matsuyama, Japan*)

In this paper is presented the isolation procedure of nucleic acids from rice plant, which is found through some modifications of the phenol method of Schramm. The nucleic acid preparation obtained by this procedure has the following some characteristic properties as RNA:

(1) The value of N-P ratio of the sample ranges around 1.64 and it is almost equivalent to the ratio of the yeast RNA examined. No amount of protein can be measured by any method.

(2) The sugar component of the sample is estimated as ribose after application of the Mejbaum's and the Dishe's reactions after paper chromatographic technique.

(3) The purine and pyrimidine bases derived from the sample are found to be closely resemble to the standard bases. And the ultraviolet extinctions of the mononucleotides from the preparation are quite similar to those of the yeast RNA examined. The value of purine-to-pyrimidine ratio is 1.0 to 1.3 or the value of A+U/G+C is 0.9 to 1.2.

(4) The ultraviolet absorption spectrum of the sample presents maximum at 258 mμ, e(P)=8500, and minimum at 230 mμ, e(P)=3800, in pH 7.0; and appears to be closely similar to that of the yeast RNA examined.

From the results obtained, the nucleic acid preparation examined can safely be called ribonucleic acid (RNA) with high purity and homogenity from the chemical and physicochemical point of view.

Studies on the Effects of Some Physical Conditions on the Submerged Mold Culture. Part III. Relations between the Morphological Forms of Molds and the Viscosity of Mycelial Suspensions. (p. *100～103*)

By Jōji TAKAHASHI and Kōichi YAMADA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

It is expected that the viscosity of mycelial suspension increases according as morphological form of suspended mold becomes more filamentous and more homogeneous.

From this point of view, a new expression of morphological form of mycelium in the culture fluid are examined by the determination of the viscosity of mycelial suspension, instead of the usual expressions such as pellet or pulp form.

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
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Studies on the Dehydration of Juices. Part I. Effects of Salts on the Puff-drying of Juices by Vacuum Dehydration. (p. *105~112*)

By Satohiko HATTORI

(*Takasago Perfumery Co., Ltd.*)

A study was made on the vacuum drying of citrus concentrated juices; Unshiu and Natsudaidai using the vacuum shelf dryer. The experiments of dehydration were carried out on plastic film loading of about 1 kg natural solid per square meter under 35°, 45° and 55°C production temperature, 0.5 mm Hg pressure and 2.5 hours drying period.

The following results were obtained.

(1) The open, sponge like structure as an aid to rapid drying could be produced by Unshiu juice, but not by Natsudaidai.

(2) Among the various puff-drying aid for Natsudaidai juice, $Ca(OH)_2$, $CaCO_3$ and calcium citrate gave satisfactory results.

(3) The amounts of these salts required are only half sufficient to produce monocalcium citrate by combining the total citric acid of concentrated juice.

Studies on the Dehydration of Juices. Part II. Changes during Drying and Storage. (p. *112~116*)

By Satohiko HATTORI

(*Takasago Perfumery Co., Ltd.*)

In the previous report (I) the effect of additives on puff-drying of citrus juices; Unshiu and Natsudaidai in the vacuum shelf dryer has been reported. In this paper, the chemical and organoleptic changes occurring during the vacuum drying process and storage period are described. Samples were stored at room temperature, 28°C and 38°C for one year. No measurable loss of ascorbic acid occurred in the drying process even at raised temperatures. In-package desiccation using CaO to reduce moisture content of samples to below 1% during storage was found to be very effective in reducing flavor deterioration, undesirable color changes, sticky mass formation, and loss of ascorbic acid during storage at raised temperatures.

Filling of dry nitrogen into cans had no significant effect on stabilization of the powders. It was further found that the storage stability of Unshiu powder is slightly greater than that of Natsudaidai.

A New Butanol Producing Anaerobic Nitrogen Fixer, *Clostridium aurantiacum* nov. spec. (p. *117~121*)

By Tsuneji SUTO, Kenji KURASHIMA and Midori MATSUKI;

(*Faculty of Agriculture, Tohoku University, Sendai*)

Yasunosuke NAMBA and Choseki FURUSAKA

(*Institute for Agricultural Research, Tohoku University, Sendai*)

From the soils of pots planted with rice in the green house of the constant temperature (27° C), a red-orange pigment producing anaerobic nitrogen fixer was isolated in the midsummer. The organism produces mainly butanol, subordinately ethanol and scarcely aceton and volatile fatty acids in the nitrogen free medium under the atmosphere of nitrogen.

The organism belongs to one of *Clostridium* species. The young cells are rods of 1–2 μ 5–6 μ and when old they trun to the spindles in shape and contain a terminal ellipsoidal spore. It grows rather better in the medium containing combined nitrogen.

It assimilates and ferments glucose, fructose, mannose, maltose, sucrose, pectin, glycogen, inulin, and starch, producing much slime substance and red-orange pigment, but does not xylose, arabinose, rhamnose, lactose, glycerol, ethanol, acetone, acetate, lactate, succinate. In the milk the spongy clot is formed slowly and fermentation scarcely occurs. Gelatin is liquefied but coagulated egg-white is not digested. The organism grows well on the potato slant without digestion of the potato.

According to the classification of Bergey's Manual and that of Prévot's this strain resembled to *Cl. chromogenes*, *Cl. roseum*, *Cl. felsineum*, *Cl. flavum* and *Cl. aurantibutyricum*. But this organism differs mainly from the following points, i.e., potato liquefication dye production on potato slant and utilization of pentose and lactose. As this organism is distinguishable from the organisms authentically described and recognized as a new species and propositionally named *Clostridium aurantiacum*.

Effect of Vitamin B_{12} on the Metabolism of Pyruvate in the Rats. (p. *122~128*)

By Junzo HAYASHI
(*Kyoritsu Women's University Junior College, Kanda-hitotsubashi, Tokyo*)

Studies were made on pyruvate oxidation system in vitamin B_{12} deficient rats. The enzymatic activity was estimated by evolved of CO_2 in the presence of pyruvate, into a washed liver homogenate and unwashed.

Qo_2 (N) and Qo_2 (N) values of B_{12}– deficient homogenate was lowered control. The addition of B_{12} in homogenate of deficient group was not increased up-take of O_2 and glutathione was added with medium stimulated. This stimulation was showed in the normal rat, too.

The oxidation of pyruvate in B_{12}-deficient homogenate was stimulated by added coenzyme (DPN, CoA, LiA and DPT), yet was lowered than normal homogenated.

The results seem to lead to the conclusion that vitamin B_{12} contributes to the activation of the apoenzyme for the formation of pyruvate oxidation system in rats.

Studies on Bacterial Lysozyme. Part I. Mode of Actions to Cell Wall Polysaccharide. (p. *128～132*)

By Sigetaka OKADA and Juichiro FUKUMOTO
(*Institue of Polytechnics, Osaka City University*)

Mode of actions of bacterial lysozyme has been observed in the culture fluid of a certain *Bacillus subtilis*.

As a substrate of it polysaccharides were prepared from the cell walls of *Micrococcus lysodeikticus*. And a few properties i.e. bacterial lysozyme's degradation ability for the polysaccharides, the characteristics of the end products formed by the treatment of it with them and etc. were pursued. In order to make clear a part of the mechanism of the break-down, the same procedures were conducted with the use of egg-white lysozyme and both properties were compared.

Following results were gained.

(1) The end reducing value by the treatment of bacterial lysozyme with the substrate showed always lesser reducing power than that by egg-white lysozyme, though, the value was slightly varied according as each way of preparating substrate.

(2) It was found that the components of the end products by egg-white lysozyme were mainly two saccharides; one of them consists of glucosamine and glucose, the other of glucosamine and unknown amino sugar. On the other hand the products decomposed by bacterial lysozyme consist of mostly, a saccharide which has two units of glucosamine and unknown amino sugar.

Studies on Bacterial Lysozyme. Part II. Muramic Acid and Lysozyme Sensitivity. (p. *132～135*)

By Sigetaka OKADA and Juichiro FUKUMOTO
(*Institue of Polytechnics, Osaka City University*)

There are two main components in the oligosaccharide of bacterial cell wall by treatment with bacterial lysozyme. They are glucosamine and unknown aminosugar. It could be considered that unknown aminosugar was muramic acid strage insisted, by Elson-Morgan color test, electric properties, paperchromatography and etc..

Furthermore, the following fact was found by detecting the quantity of muramic acid and lysozyme senstivity with use of cell wall polysaccharide of *B. subtilis* var. *amyloliquefacience* F., parts with high quantity of total amino sugar were more split by egg-white lysozyme and only the fraction with high quantity of muramic acid of it was broken down by the bacterial lysozyme.

Evidence for this was confirmed in the case of other several bacterial species by the comparison of two properties of cell constractive aminosugar and degree of lysis by lysozymes. It could be considered higher specificity of bacterial lysozyme than that of egg-white.

Studies on Organic Acid Metaboism by *Rhizobium japonicum*. Part I. Behaviour toward Several Organic Acids and Oxidation of α-Ketoglutaric Acid. (p. *136～139*)

By Yukihiko NAKAMURA, Tokuji SHIMOMURA and Ko SAWAI
(*Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University*)

I. Growth of *Rhizobium japonicum* was very well in the culture media containing ammonium chloride and potassium nitrate, to which each intermediate on TCA cycle, adipate, glutarate or tartarate was added as carbon source respectively, but is poor on addition of formate, acetate, butyrate, valerate or isovalerate. By the use of washed cell suspensions of this organism grown on the other media consisting of mannitol, glutamate and several inorganic salts, oxygen consumption was determined manometrically in the presence of each of these acids as substrate, and the reduction time of methylene blue was measured by Thunberg's method. The value of oxygen uptake (μl)/mg N/hour (38°C) decreased in the following order: α-ketoglutarate, 186; succinate, 185; glutarate, 143; lactate, 101; malate, 99; fumarate, 98; pyruvate, 75; other acids, 50 or below. Methylene blue was decolorized more rapidly by the intermediate on TCA cycle than by the other acids. From these results, it was assumed that TCA cycle system might exist in this organism.

II. The crude enzyme preparation catalyzing the oxidation of α-ketoglutarate was obtained from bacteria grown on the mannitol media. After one hour's reaction period, 1 mole of carbon dioxide was produced and 0.5 moles of oxygen taken up, and succinate was identified as the reaction product. Therefore the existence of the α-ketoglutarate oxidizing enzyme was certain in bacteria, which seemed to catalyze the following reaction:

α-ketoglutarate$+1/2\ O_2 \longrightarrow$ succinate$+CO_2$.

Chemical Studies on Toxic Protein "Ricin" in Castor Bean. Part IV. The Chromatographic Fractionation of Crystalline Ricin. (p. *139~142*)

By Gunki FUNATSU

(*Biochemical Laboratory, Faculty of Agriculture, Kyushu University*)

Crystalline ricin was fractionated two fractions (ricin C_1 and C_2) by means of calcium phosphate column chromatography using phosphate buffer. Ricin C_1 was the real toxic protein which had no proteolytic and hemagglutinating activities, and the simple protein which was homogenous ultracentrifugally and electrophoretically.

Hydrolysis of Protein modified with UV Light Irradiation by Proteases. (p. *142~145*)

By Mitsuo KOSHIKA and Yataro OBATA

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α-Casein and egg lysozyme were divided into four fractions, namely, 1) UV light irradiated, 2) non-irradiated, 3) heated and 4) irradiated-heated fractions. These protein fractions were digested by crystalline trypsin, chymotrypsin and pepsin and the amounts of increased carboxyl group were estimated periodically.

Irradiated α-casein and lysozyme were less susceptible to these proteases than native protein. When irradiated and non-irradiated proteins were heated at 90°C for half an hour, the susceptibility to the enzymes both were elevated, especially, the digestibility by trypsin was not different from heated and irradiated-heated proteins. However, the digestibility of irradiated-heated protein by pepsin or chymotrypsin was still lower than the only heated protein.

Effect of Urea on Casein in Milk in the Process of Rennin Coagulation. (Studies on Milk Coagulating Enzymes. XVII) (p. *145~150*)

By Kunio YAMAUCHI

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Experiments were performed on the effect of adding urea on skim milk (A), skim milk treated with rennin but not coagulated yet (B) and skim milk coagulated with rennin (C). Into 10 ml of skim milk or curd was added an adequate amount of concentrated urea solution after a given time from addition of rennin and the volume was adjusted to 20 ml with water. The amount of precipitated protein was determined after centrifugation. C was dissolved at 6 M and higher concentration of urea and transparent solution was resulted. A became transparent with increase of urea concentration. No difference between A and C dissolved in concentrated urea solution was found as to pH and viscosity.

Effect of urea on B was different from that on A and considerably complicated. When relatively low concentration of urea (3.33 M) was added into B, coagulation appeared rapidly. The amount of the coagulum formed increased and the time required for the coagulation after addition of urea decreased with the progress of rennin action respectively. This suggests that intra-micelle reaction of casein in milk proceeds progressively by rennin before curdling occurs. It seems that casein particle in B, which is yet stable, is once dissociated by urea and then the other association of the casein which has become reactive by rennin occurs. This coagulum was redissolved at higher urea concentration. The ratio of α- to β-casein in the coagulum was almost the same as that of ordinary unfractionated casein.

Chromatographical Observation of γ-Irradiated Potato Starch Granules. (p. *150~153*)

By Akira MISHINA and Ziro NIKUNI

(*Institute of Scientific and Industrial Research, Osaka University*)

From the highly irradiated product of potato starch granules, glucose, maltose, arabinose, glucuronic acid, gluconic acid and a series of small dextrins were identified by paper chromatographic analysis. These mono- and oligosaccharides were analyzed by the anion exchanger column chromatography, and glucose and maltose were determined quantitatively as main fission products.

A gas chromatography was also carrid out to analyze the gaseous product of starch granules by γ-radiation, and hydrogen carbonmono- and dioxide were identified.

From these results, a part of the mechanism of degradation of starch by γ-radiation was discussed.

Studies on L-Valine Fermentation. Part I. Production of L-Valine by *Aerobacter* Bacteria. (p. *153~158*)

By Zenjirô SUGISAKI

(*Noda Institute for Scientific Research*)

Those studies have been undertaken with the object of fermentative production of optically active L-valine.

A screening test for valine producing bacteria was carried out, and it was recognized that in shake culture a rather large amount of valine is practically produced as the sole amino acid in the culture broth from carbohydrates and nitrogen sources by some species of bacteria.

Two strains of newly isolated L-valine accumulating bacteria were studied according to the system described in Bergey's manual, and named *Aerobacter cloacae* var. sp. NISR-B-151 and *Aerobacter aerogenes* NISR-B-801, respectively. The high level of L-valine production is attributed to the newly isolated *Aerobacter* bacteria, yielding as much as 0.2 mole from one mole of glucose.

L-Valine was isolated from the culture broth in the crystalline form and identified.

Studies on L-Valine Fermentation. Part II. Factors Affecting Production of L-Valine. (p. *158~165*)

By Zenziro SUGISAKI

(*Noda Institute for Scientific Research*)

External factors affecting fermentative production of L-valine were investigated and optimal conditions were determined for the shaken flask production by *Aerobacter aerogenes* NISH-B-801.

It was comprehended that optimal C/N-ratio (C/N= 100 : 78; cf, glucose 10% and NH_4Cl 1.2%) and adequate aeration were leading factors to bring about L-valine fermentation, and moreover inorganic ingredients of medium, not only bulk elements but also trace elements like Ni^{++}, Mn^{++}, and Mo^{+6}, had great influences on valine formation. The most efficient fermentations were performed with limited but adequate growth of the speciffc bacteria under the optimal pH in region from 5.4 to 6.2.

Studies on the Citric Acid Production with *Trichoderma viride*. Part I. Studies on their Isolation, Identification, Distribution and Citrogenicity. (p. *166~170*)

By Osamu TERADA, Kazuo OHISHI and Shukuo KINOSHITA

(*Tokyo Research Laboratory, Kyowa Fermentation Industry Co.*)

A culture of *Tichoderma viride* isolated from dung of *Muntiacus reeversii micrurus* was found to produce citric acid from glucose or starch. The yields of citric acid were from 60 to 85% of theory to initial carbohydrates, and the presence of calcium carbonate in culture media was found indispensable for high yields.

No concomitant acids were found. The productivities of citric acid were investigated on number of isolates from forest soils and cultures with high citrogenicity were found to distribute in fairely high frequency in nature.

Studies on the Utilization of Hydrol. Part I. The Production of *d*-Saccharic Acid by the Nitric Acid Oxidation of Hydrol. (1) (p. *170~173*)

By Sigeo KIYOOKA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Systematic experiments were carried out to find the best condition of the nitric acid oxidation of hydrol to *d*-saccharic acid under the following conditions. Nitric acid concentration: 30~62%, corresponding 3~6 moles per mole of dry substance of hydrol, reaction temperature: 55~93°C, and the reaction time: 7 minutes~3 hours. On the results of the experiments, the best condition of the oxidation was found at higher temperature and lower acid concentration than in case of glucose, and the maximum yield of *d*-saccharic acid was 31.2% of the theoretical based on dry substance of hydrol.

Studies on the Utilization of Hydrol. Part II. The Production of *d*-Saccharic Acid by the Nitric Acid Oxidation of Hydrol. (2) (p. *173~177*)

By Sigeo KIYOOKA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Hydrol was oxidized to *d*-saccharic acid (I) by nitric acid, using potassium nitrite or sodium meta-vanadate as catalysts.

1. In the case of potassium nitrite, the reactions were carried out under the following conditions. Nitric acid: 30~60%, corresponding 3~4 moles per mole of dry substance of hydrol, potassium nitrite: 1~7%, reaction temperature: 16~93° C, and the time: 2~88 hours.

By addition of nitrite, the yield of I did not increase, but did not decrease even in lowering the acid concentration or the reaction temperature. The maximum yield of I was 30.8% of the theoretical based on dry substance of hydrol.

2. In the case of vanadate, the reaction conditions were as follows.

Nitric acid concentration: 43~60%, corresponding 4~6 moles per mole of hydrol. Sodium meta-vanadate

(tetra-hydrate) 0.001～0.1% of hydrol. Reaction temperature: 25～85°C, time: 0.7～88 hours.

Under a condition of 4 moles of nitric acid and 0.001 % vanadate, the yield of I was 28～29%, but when the vanadate was added in the range of 0.005～0.01%, the yields decreased to 11～24%, and 10～17% dextrotartaric acid (II), 7～26% oxalic acid (III) and a very small amount of racemic acid (IV) were found. If 0.1 % of vanadate or 6 moles of nitric acid was used, I were 1～3%, II 3～5%, III 5～12% and IV 1～10%.

X-Ray Diffractometric Studies on Starches. Part V. Relation between Viscosity Change and Micelle Structure in the Gelatinization of Starches.

(p. *178～182*)

By Susumu HIZUKURI, Mitiko FUJII and Ziro NIKUNI

(*The Institute of Scientific and Industrial Research, Osaka University*)

Aliquots of starch suspensions or pastes were withdrawn from a V. I. Viscosigraaf vessel during viscosity measurements corresponding to the several specific points of their viscograms. These specimens were treated with alcohol and the dried specimens were examined by X-ray diffraction method. V.I. Viscograms of the examined starches were divided into 2 groups. Group I (potato, sweet potato, defatted corn, defatted rice, waxy corn and waxy rice starches) showed a big viscosity tangent and group II (non-defatted corn and rice starches) gave a small viscosity tangent.

At the starting points of the rapid elevation of viscosity curves, the X-ray diffraction curves of the ordinary starch specimens (both in group I and II) diffused already showing that their micelle structure was destroyed seriously. But waxy starches retained their micelle structure better.

In group I, these original β-starch X-ray spectra were slightly observed at the half of the maximum viscosity, then they disappeared completely at the maxim viscosity point (η_{max}). In group II, however, the original β-spectra disappeared completely even at the half of the η_{max} and weak helical spectra appeared.

Thus the viscosity change and the structural change of starch granules were not parallel between starch speciinens.

Biochemical Study of Rice Starch. Part XII. Isomylase Fraction in Seedrice at the Milky Stage.

(p. *183～187*)

By Humio KURASAWA, Tosiro HAYAKAWA and Ikuo IGAUE

(*Department of Agriculture, Niigata University*)

We have prepared the isoamylase fraction from the seedrice at the milky stage. Precipitation of isoamylase fraction was obtained by adding ammonium-sulfate (18 ～25 g/100 ml) to the extract of the seedrice at the milky stage.

1. After isoamylase fraction was added to amylopectin it was found by an iodine reaction test that the normal violet color of amylopectin changes gradually to blue violet.

2. The optimum temperature for the activity of this fraction is about 40°C.

3. The optimum pH is about 5.0～5.5.

4. When an isoamylase fraction is added to amylopectin, the viscosity of the reacted solution decreases to the original amylopectin solution.

5. The electro motive force of the reacted solution (by using potentiometric method) becomes lower than the original amylopectin solution.

6. When this enzyme fraction is added to an amylopectin solution for 24 hours, 88 per cent of glucose is produced from amylopectin.

Studies on Shinshiki Shoyu (Semi-Chemical Soy Sauce). Part I. On the Change of General Components in the Cource of Manufacture. (p. *187～193*)

By Takahiro UENO and Taira KURAMOCHI

(*Noda Soy Sauce Co. Ltd.*)

1. The general components in the hydrolysate of defatted soy bean with diluted (6～8%) HCl were measured. At the end point of hydrolysis, the contents of reducing sugars and furfural were higher than those observed in chemical soy sauce (the concentrated HCl hydrolysate). After neutralizing at 80°C, the contents of amino-N, reducing sugars and pH value decreased gradually but the color formation and the decrease of furfural content were remarkably high. L-Glutamic acid content decreases approximately 10% by heating for 48 hours at 70°C, pH 5.0.

2. L-Glutamic acid was heated with 5% NaCl and 2～8% HCl for 0～8% hours at boiling point. By heating the solution of L-glutamic acid alone, the decrese of glutamic acid content in 5% NaCl and 6～8% HCl were little, but in 1～2% HCl, those were large, due to the pyroglutamic acid formation.

By heating the solution of L-glutamic acid in the presence of xylose, glucose and furfural, the color formation and the decrease of glutamic acid content in 5% NaCl were very large, but in the HCl solutions, those were almost similar to glutamic acid and reducing sugars alone respectivelly.

(*continued from front cover*)

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